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COMMISSION ON EPIDEMIOLOGICAL SURVEY

ARMED FORCES EPIDEMIOLOGICAL BOARD

ANNUAL REPORT - 1963

COMMISSION ON EPIDEMIOLOGICAL SURVEY

Armed Forces Epidemiological Board

ANNUAL REPORT

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Wisseman, Charles L., Jr., M.D.****

* Deceased 31 July 1963.
** Resigned 1963.
*** Elected to Full Membership December 1963.
**** Elected to Associate Membership December 1963.

THE DIRECTOR'S REPORT

The Commission on Epidemiological Survey held its annual meeting at the Walter Reed Army Institute of Research on August 28 and 29, 1963. Several Ad Hoc meetings for discussion of specific problems were held during the year. In addition to senior representatives from the Departments of the Army, Navy, Air Force, the U. S. Army Medical Unit and the U. S. Army Biological Laboratories at Fort Detrick, there were other guests in attendance. These included Dr. I. H. Lepow, Western Reserve School of Medicine and Chairman of the Commission on Immunization, Dr. Kenneth Goodner, Professor and Head, Department of Microbiology, Jefferson Medical College and Member, Commission on Immunization, and Dr. J. E. Johnson of the Johns Hopkins School of Medicine. Captain Sidney A. Britten, USN, was welcomed as the new Executive Secretary, successor to Colonel Moseley. The Director acknowledgedwith gratitude the efficient manner in which Colonel Moseley and Miss Gilbert helped administer the affairs of the Commission. A special tribute was paid to Dr. Joseph E. Smadel, a pioneer Member of the Commission and one of the nation's top scientists, who died on July 21, 1963. In a brief Memorial Minute, Dr. Smadel was described as a painstaking investigator, an inspired leader, a dedicated friend of this Commission and the Armed Forces Epidemiological Board. An excerpt of a tribute paid Dr. Smadel on December 9, 1963, before the Armed Forces Epidemiological Board reads:

"The Members of this Board knew Joseph E. Smadel as one of its ablest, scientifically active contributors extending over two decades. He organized and directed the programs of two Commissions, Immunization and Rickettsial Diseases, and stabilized the Commission on Epidemiological Survey during its formulation and expansion. Achievements of these Commissions have made mankind healthier, richer, and each proudly bears an indelible Smadel mark."

The resignations of Dr. Richard E. Shope and Dr. Colin M. MacLeod were accepted with great regret. Dr. Shope, a Charter Member and initial Director, inspired and guided a distinguished scientific program. The Commission, under him, developed a broader understanding of the basic mechanisms of certain infectious diseases and effective means of their control. Administrative difficulties he took in stride. Now wishing to devote his full time to the scientific program of the Rockefeller Institute, he will be called upon for advice and help.

Dr. MacLeod was forced to resign because of heavy responsibilities as Executive Director of the President's Scientific Panel. Through many years he responded to appeals for advice which made seemingly impossible problems simpler and workable. Unfailing in his ready assent to serve, he did so on short notice without regard to personal inconvenience. Dr. MacLeod contributed mature judgment and broad vision sorely needed by the Commission during more than a decade of service.

It is comforting to have him close at hand for help. Any scientific group privileged to have had the continuing guidance of men such as Smadel, Shope and MacLeod is unique. The scientific and intellectual stimulation provided by them is proudly acknowledged.

The first day's meeting was devoted to hearing reports of work completed or in progress by investigators of the U. S. Army Medical Unit and contractors.

Dr. Martha Ward reported on simplified methods of concentrating anthrax toxin and indicated that an ultrafiltrate maintains its activity in a frozen state.

Colonel Irving Gray discussed the development of fluid loss, pulmonary edema, hemoconcentration, and increase in weight of lungs and kidneys in rats following administration of anthrax toxin. His findings reveal resistance to the lethal effects of Bacillus anthracis in rats fed normal diets and decreased resistance in lysine deficiency. Studies of leukocyte activity by the Rebuck technique reveal a smaller leukocytic response in gluten fed rats with increased deaths following anthrax administration. The data are not regarded as conclusive. Although anthrax toxin has not been characterized fully, good progress was reported.

Lt Colonel W. R. Beisel described results of metabolic studies in human subjects during the stress of specifically induced infection, i.e., tularemia. Urinary excretion of nitrogen and steroids increased with steroid levels rising before the maximum temperature level. Immunized subjects showed little change in steroid levels. Urinary uric acid excretion was not increased. There were negative phases of Na, K, and Cl which correlated with temperature elevation. Loss of intracellular components of phosphate, potassium, magnesium, and nitrogen continued into convalescence. These latter findings were statistically significant.

A technique described by Mr. Robert Jaeger, using immunochemical techniques, identified the virus of Venezuelan equine encephalomyelitis in tissue culture cells within four hours after infection.

Typhoid fever studies in volunteers were discussed by Doctors Richard B. Hornick and Sheldon E. Greisman of the University of Maryland School of Medicine. Based on the observations of approximately one hundred volunteers, the ID_{50} dose of Salmonella typhosa (Quailes strain) is approximately 10^6 organisms. Of the four typhoid vaccines employed prophylactically, the acetone preparation shows a tendency to protection at the challenge dose specified. These data are preliminary. Eight volunteers previously infected and made ill with typhoid fever developed a second attack when rechallenged following ingestion of 10^7 viable bacterial cells. The newer antibiotics, Ampicillin, Humatin, Colymycin, and Gentamycin have failed to equal chloramphenicol in therapeutic effectiveness when tried in a limited number of infected patients.

Dr. Hornick reported that the continuous daily administration of a therapeutic dose of chloramphenicol for twenty-eight days beginning with infection successfully prevented active clinical manifestations. These subjects developed serologic response but no clinical disease. Seven days chemoprophylaxis failed to prevent active infection in two volunteers.

Dr. Greisman's physiologic studies continue to show that typhoid patients develop tolerance to endotoxin in the early convalescent phase of the disease (third afebrile disease). Strikingly, tolerance to intravenously infused endotoxin falls during active infection and just before the febrile phase. During the late incubation period and the active febrile phase, volunteers are able to clear Cr^{51}-labeled endotoxin at an accelerated rate. The concept is entertained, and under study, that tolerance is related to immunologic mechanisms such as the action of opsonins. Conceivably, during active infection, such mechanisms are paralyzed and artificially infused endotoxin is free to act directly on target centers. These studies have been published in various journals.

Dr. Hugh B. Tresselt reported the growth of fully virulent SCHU-S4 strain of Pasteurella tularensis on blood-free media consisting of ferrous sulfate and histidine as a substitute for blood in basal media.

Colonel Raymond Randall described the favorable effects of the Rift Valley fever vaccine and reported that 7,000 ml had been administered to humans without ill effect or significant side reactions.

In his report of studies of plague antigens, Major J. D. Marshall reported the fractionation of Pasteurella pestis by chromatographic methods into components containing two to seven antigens. Studies are designed to develop and evaluate an effective vaccine against pneumonic plague.

The second day's meeting was devoted exclusively to hearing reports and a general discussion of the studies of staphylococcal enterotoxin by the U. S. Army Medical Unit. These results are reported and summarized elsewhere.

Theodore E. Woodward, M.D.
Director

February 10, 1964

RECENT PROGRESS IN ANTHRAX STUDIES

Martha K. Ward, Captain, USPHS*

The work I will report has been in two general areas: (1) Studies on the characterization of various strains of Bacillus anthracis which differ in their biological activity; and (2) Studies on anthrax toxin. A number of people have contributed to this work, so to avoid possible omissions of proper credit as I go along, I should like to point out now that I am acting mainly as recounting for several co-workers and colleagues. The first section summarizes work done by Dr. McGann and Mrs. Roberts. Section II summarizes work of Dr. Tresselt, Commander Gaspar, Mr. Kanode, Dr. Buzzell and Colonel Gray.

I. Characterization of Various Strains of Bacillus anthracis which differ in their Biological Activity.

Interest in studies on characteristics of various strains of B. anthracis has been stimulated by several observations made during the course of other work with anthrax over the past several years; first, the isolation of penicillin-resistant organisms from an animal inadequately treated with the antibiotic; secondly, the corroboration of earlier work by Auerbach and Wright[1] who demonstrated that vaccination of guinea pigs with anthrax protective antigen afforded protection against challenge with certain strains of anthrax but not against others. These strains which differ so markedly in their in vivo activity could not be readily distinguished from each other by any in vitro methods available. In addition, a number of "atypical" organisms were isolated at autopsy from immunized animals that succumbed to challenge during our studies on the efficacy of the vaccine.

Blood agar plates inoculated with a sample of spleen taken at autopsy of a monkey dying from anthrax infection after treatment with penicillin contained some typical colonies of B. anthracis, but dwarf colony forms predominated. Growth of the small colony forms was slow on standard media and many cells were abnormal in shape, size or staining characteristics. Sporulation was poor, and plating of spore suspensions yielded several different colony types. Techniques for producing homogeneous spore suspensions of encapsulated "antibiotic-resistant" mutants were developed, and suspensions representative of a variety of cultural characteristics were prepared. Some of these were essentially identical to the parent strain except for antibiotic "resistance," whereas others were markedly different.

Experience with these "antibiotic-resistant" strains was helpful when, in the course of the studies on immunogenicity of anthrax protective

* U. S. Army Medical Unit, Fort Detrick, Maryland.

antigen, we recovered several colonial types from immunized guinea pigs that had succumbed to challenge with strains V1b-189 or NH6. In these studies the use of bicarbonate agar for direct plating enabled us to distinguish rapidly between typical capsulated forms and occasional colonies of noncapsulated organisms. We were also able to detect on this medium 3 types of capsule-forming colonies: (a) those typical of the parent strain; (b) dwarf or pinpoint and, (c) a type of normal size but thick and opaque.

Recovery of these colonial types from blood and/or spleens of moribund guinea pigs is summarized in Table I. Except for a few samples with an

TABLE I. INCIDENCE OF MUTANTS OF B. ANTHRACIS IN BLOOD AND/OR SPLEENS OF MORIBUND GUINEA PIGS

	CHALLENGE STRAIN AND GROUP			
	V1b-189		NH6	
	Nonimmunized	Immunized	Nonimmunized	Immunized
No. with satisfactory plates	25	29	16	30
No. with colony type typical of parent	25	25	16	29
Mutants: total	0	15	0	7
Pinpoint	0	9	0	4
Opaque	0	5	0	3
Rough	0	1	0	0
Percent animals with mutants	0	52	0	23

occasional rough colony, no mutants were recovered after challenge of nonimmunized guinea pigs. Significant concentrations of mutant types were found, however, in 35% of the immunized animals that succumbed to challenge. Approximately 20% of these contained pure populations of mutants; the remainder had mixed populations of mutant and typical colonies. The results suggested that mutants appeared more frequently after challenge with strain V1b-189 than after challenge with NH6. Incidence of mutants was not related to prechallenge antibody titers, but appeared to increase with survival time, especially after challenge with strain V1b-189.

Cultures were tested for homogeneity, and spore suspensions were made. Cultural characteristics and virulence for guinea pigs were determined for 10 of these suspensions and also for 2 of the "antibiotic-resistant" mutants isolated from the penicillin-treated monkey.

Some cultural characteristics and relative virulence of parent strains and selected mutants are shown in Table II. In general, all mutants were

TABLE II. VIRULENCE AND CULTURAL CHARACTERISTICS OF PARENT AND MUTANT STRAINS OF B. ANTHRACIS

PARENT STRAIN	MUTANT STRAIN	GUINEA PIG LD_{50} (Spores)	COLONY TYPE	PENICILLIN RESISTANCE	CLONE LYSIS (Minimal Medium)	DELAYED HEMOLYSIS ON SHEEP BLOOD AGAR PLATES
V1b-189	-	4	Somewhat opaque	-	-	Weak
	PENICILLIN					
	10-24	20	Opaque	+	-	Weak
	4-8	240	Opaque, yellow	+	-	-
	IMMUNE					
	201A	10	Opaque	-	-	Weak
	203C	15	Opaque, rimmed	+	+	Strong
	5C	20[a]	Pinpoint	-	-	-
	205A	35	Opaque, creamy	+	-	-
	202B	50[a]	Pinpoint	-	-	Weak
NH6	-	35	Translucent	-	+	-
	IMMUNE					
	114C	9	Opaque	+	+	-
	101C	11	Translucent	+	+	-
	105C	16	Opaque	+	+	Weak
	308C	15	Opaque, small	+	+	Variable
	101A	50[a]	Opaque, creamy, yellow	-	-	Variable

a. Long chains of organisms in spleen impressions.

more opaque and grew more slowly than did the parent strains. All cultures were encapsulated when grown on bicarbonate agar in an atmosphere of CO_2. Two of the V1b-189 mutants (5C and 202B), however, did not grow under these conditions unless vegetative cell inocula or high concentrations of spores were used. Several mutants were resistant to penicillin although they had not been previously exposed to the antibiotic. On a minimal synthetic medium colonies of the parent culture and of most mutants of the NH6 strain appeared eroded or lysed, as if by bacteriophage.

In every instance, characteristics of organisms recovered after death were the same as those of the corresponding challenge suspensions. It was interesting to note that after challenge with 2 of 5 strain V1b-189 "immune" mutants and 1 of 5 strain NY6 mutants, long chains of organisms were found in spleen impressions and that the relative concentration of bacilli was lower

than in animals challenged with the parent strain. Organisms in spleen impressions from animals challenged with all other mutants were in the usual short chains.

Unfortunately, at the present time there are no generally recognized or satisfactory criteria for distinguishing among strains of B. anthracis, except capsule production. As an approach to this problem, specific requirements for germination and growth from spore inocula (250 spores/ml) of several representative strains are under investigation using chemically defined media. As others have demonstrated, there were few requirements for optimal germination. Addition of components that would support growth, however, often delayed germination and, conversely, compounds required for germination inhibited growth of one or more strains. Consequently, to obtain an optimal medium for outgrowth of spore inocula it was necessary to effect a combination that would minimize inhibitory activities. Some differences between the growth response of virulent and avirulent strains have been observed; however, sufficient information is not yet available to evaluate the significance of these differences.

It is proposed to continue these studies with defined media in the attempt to develop in vitro methods for distinguishing among strains differing in their in vivo behavior. It is also proposed to study the efficacy of vaccination and antibiotic therapy for protection against challenge with strains of differing in vitro characteristics, particularly those penicillin-resistant and other mutants isolated from in vivo material.

II. Studies on Anthrax Toxin.

Other workers have met with some success in the purification of the separate components of anthrax toxin using several different procedures. Although valuable information has been obtained by studies on the 3 known components separately and in recombinations, we have felt that attempts to isolate whole toxin without prior separation into its component parts would be worthwhile for several reasons.

The initial work on concentration and purification of whole toxin employed removal of organisms from the culture fluid by filtration through Millipore filters and concentration with Carbowax. This was followed by fractional precipitation with ethanol. The toxic activity as measured by rat lethality and guinea pig skin edema of culture filtrates so treated could be concentrated approximately 100-fold with total yields calculated to be between 80 and 90% of the original crude material. Unfortunately, the passage of small molecular weight components of the Carbowax through the dialysis tubing during the concentration procedure greatly increased the viscosity of the material and markedly affected and confused biophysical studies on it, although it apparently did not affect either the biological or serological activity. In addition, the process was time consuming and the 100-fold concentration appeared to be about the upper limit of the method.

Although results of this work are most encouraging so far as concentration of toxic activity for rats is concerned, results of work using guinea pig skin edema and the gel diffusion technique as methods of assay, do not follow a similar pattern. With these techniques there is evidence for significant loss of activity during the processing.

Several incidental observations we have made may be possible clues to an explanation of this apparent differential loss of activity, but as yet we do not have sufficient evidence to suggest a solution to the problem. This we hope to get in the production of future lots of concentrated toxin.

In the last two months we have begun studies on the mode of action of anthrax toxin in the Fischer rat. This work has all been done with crude unconcentrated toxin, the major purpose being that of determining which parameters might prove most fruitful areas for further investigation with the more purified material when it is available. Colonel Gray has carried the major responsibility for this work and will summarize our progress in this area.

REFERENCE

1. Auerbach, S., and Wright, G. G.: "Studies on Immunity in Anthrax. VI. Immunizing Activity of Protective Antigen Against Various Strains of Bacillus anthracis," J Immunol 75:129-133, 1955.

EFFECT OF LYSINE DEFICIENCY ON THE LEUKOCYTE RESPONSE

Irving Gray, Colonel, MSC*

The interaction between nutrition and resistance has been well established. Many reviews have summarized the current status of this problem, while more specifically Dubos has demonstrated the effect of lysine deficiency on resistance to infectious disease. In a more recent publication[1] we have related the lowered resistance to infectious disease to the activity of the fixed macrophages of the reticuloendothelial system. Inasmuch as the circulating macrophages play at least an equal role in the response of an animal to the invasion by microorganisms, this system has also been studied. The subject of this report is the ability of lysine-deficient rats to mobilize circulating leukocytes in response to a standardized irritant.

MATERIALS AND METHODS

The specifics of establishing the lysine deficiency have been previously reported.[1] Essentially, it consists of placing weanling rats on a diet complete in all known requirements, except that the protein source is 20% gluten flour. The control diets are identical, except that the protein source is 26% casein. The animals are kept on the diet for 30 days. The effect of this diet on the weight and appearance of the animals was the same as previously reported. The control animals averaged 165 gm in weight, the experimental, 80 gm. The rats used were white, females of the Sprague-Dawley strain, obtained from a commercial source.

Two techniques were used to study the mobilization of leukocytes. One was essentially that of Rebuck[2] in which his "skin-window" method was adapted for use on rats. The animals were restrained on their backs on specially designed boards. The abdomen was clipped and then closely shaved with a very sharp blade. This usually caused enough abrasion of the skin to cause the migration of the leukocytes to the site. However, to insure sufficient leukocytic activity, a site, approximately one inch square was additionally abraded with a Bard-Parker blade. A microscope slide coverslip was held in place by a ring cut from one-half inch i.d. rubber tubing placed on it and held in place and to the skin, with adhesive tape. The coverslips were replaced each hour for 8 hours. When removed, they were stained in the normal manner with Wright-Geimsa. The coverslips were then mounted on slides and examined. All preparations were coded, examined blind, and the results reported as 0 to 4+, the latter applying to those slides with the most leukocytes. An arbitrary value of 0 to 40 was given for use in evaluating the results.

The second method used to study the mobilization of leukocytes was the response to an irritant injected into the peritoneum. The material injected

* U. S. Army Medical Unit, Fort Detrick, Maryland.

was 12% sodium caseinate: 5 ml into the control animals and 3 ml into the gluten animals. After the selected time interval, the animals were anesthetized with ether, the abdomen opened, and the contents removed with a pipette. The exudates were added to 2 ml of heparinized saline, centrifuged, and the packed cells resuspended in 1 ml of saline. The total leukocyte count was determined in the standard manner.

To check the viability of the white blood cells, the preparations were stained with 0.1% eosin.

RESULTS AND DISCUSSION

Figure 1 and Table I summarize the results obtained in the "skin-window" experiments. Two facts may be deducted from these data: (a) the deficient animals do not sustain a response to a peripheral irritating stimulus to the same extent as the controls, even though the initial rate may be the same, and (b) the number of monocytes present relative to the total number of leukocytes is also reduced. When one considers that the total number of leukocytes is reduced about 30% below the control values, then the decrease in the monocytes takes on even greater significance.

TABLE I. RELATION OF MONONUCLEAR CELLS TO TOTAL LEUKOCYTES IN SKIN-WINDOW

EXPERIMENT NUMBER	ANIMALS GROUP	% MONONUCLEAR CELLS	
		Control Rats	Gluten-fed Rats
1	10	28	15
2	10	20	20
3	10	17	9
4	5	25	8
MEAN		23	13

In Figure 2 are summarized the data pertaining to the ability of the 2 groups of animals to respond to an irritant injected intraperitoneally. The results are in accord with the previous part of this paper, but are even more striking as far as the total leukocyte count is concerned. It is evident that the control animals mobilize the circulating leukocytes faster and to a higher level than do the deficient animals.

Finally, the viability of the leukocytes, as measured by the uptake of eosin by the dead cells, was the same in both groups of animals.

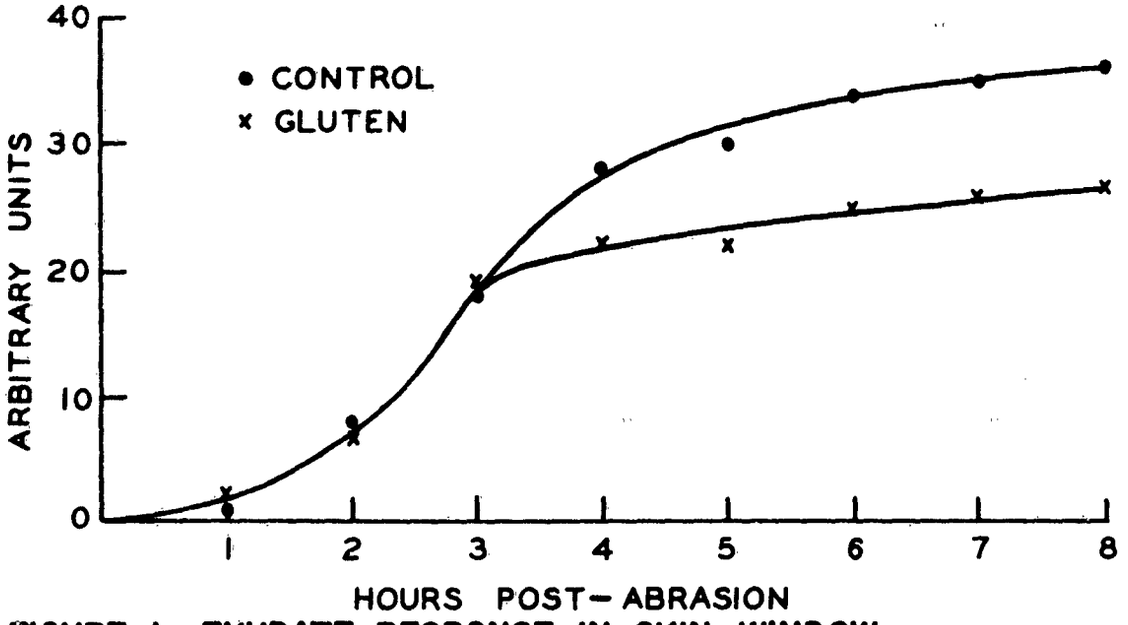

FIGURE 1. EXUDATE RESPONSE IN SKIN WINDOW.

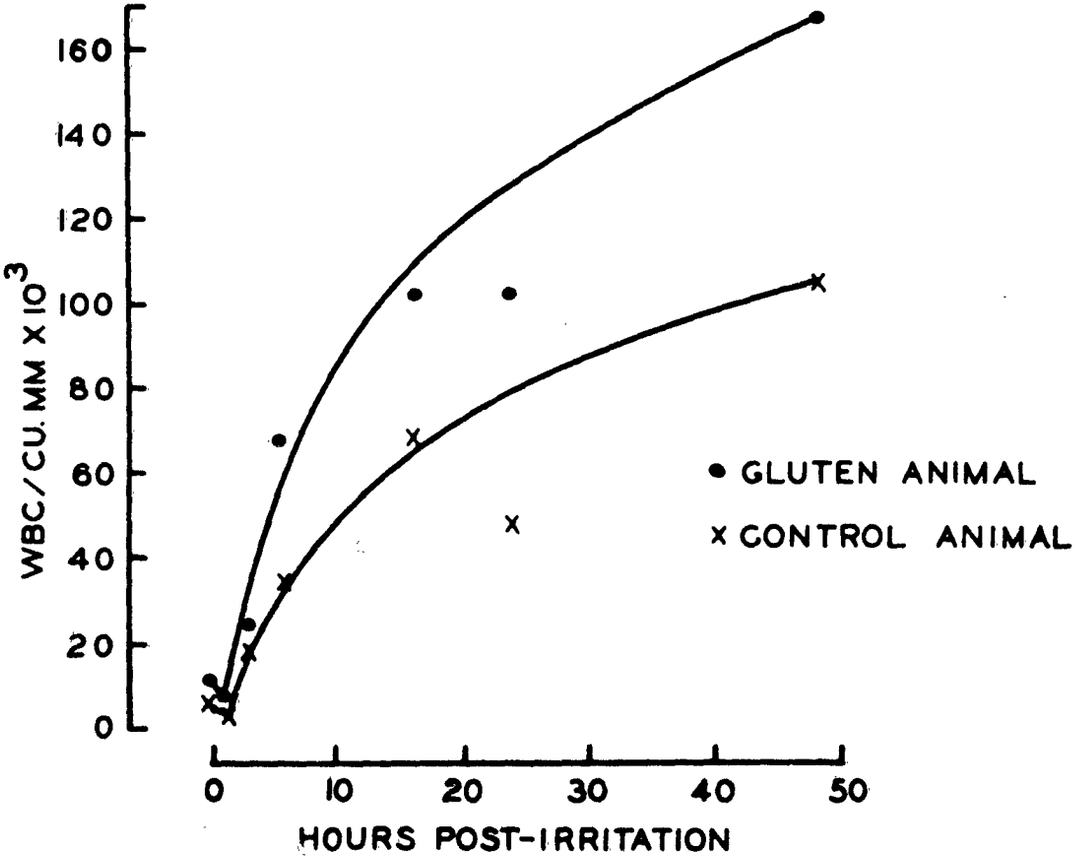

FIGURE 2. LEUKOCYTE RESPONSE TO IP SODIUM CASEINATE.

An additional study carried out to determine the phagocytic activity of the leukocytes from the 2 groups of animals was that of surface phagocytosis. A portion of this study was carried out at Dr. Barry Wood's laboratory in association with Miss Mary Ruth Smith. The ability of both groups of leukocytes to engulf Type I pneumococcus or avirulent anthrax spores was unchanged.

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1. Gray, I.: "Lysine Deficiency and Host Resistance to Anthrax," J Exp Med 117:497-508, 1963.

2. Rebuck, J. W.: "Technic for the Study of Leukocyte Functions in Man," in Methods in Medical Research, Vol. 7, J. V. Warren, Editor, Year Book Publishers, Inc., Chicago, 1958. pp. 161-164.

THE EFFECT OF ACUTE EXPERIMENTALLY INDUCED TULAREMIA IN HUMANS ON THEIR METABOLISM OF NITROGEN, ELECTROLYTES, AND MINERALS, AND ON ADRENOCORTICAL FUNCTION

William R. Beisel, Lt Colonel, MC*

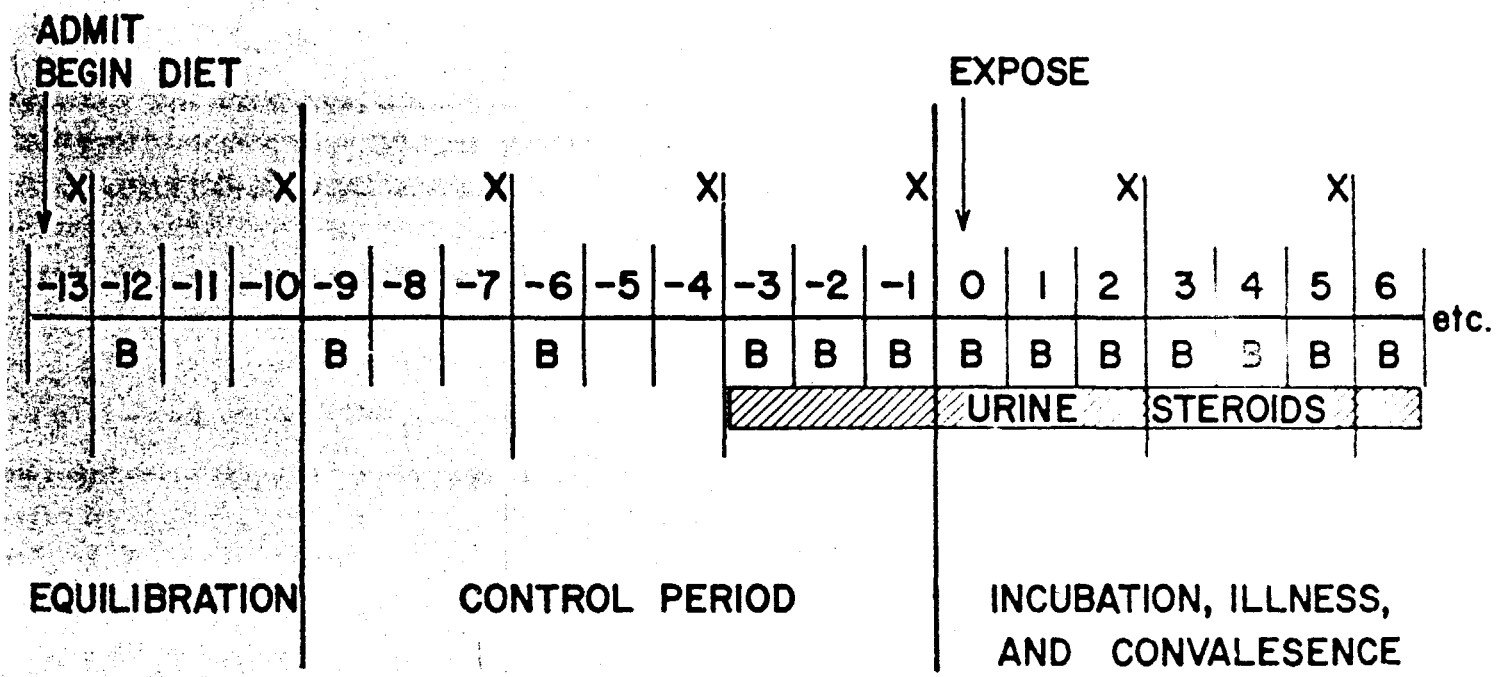
Although heightened adrenocortical function, losses of body nitrogen, and alterations in plasma electrolytes are well recognized features of an acute infectious illness, very little knowledge is available concerning complex metabolic interactions within the host during infection or their integration with endocrine responses. The majority of existing data of a metabolic nature was published prior to the antibiotic era and was obtained in patients with acute, but long lasting, febrile illnesses such as typhoid fever or pneumonia. These studies preceded the present common availability of techniques such as flame photometry, fluorometric analysis, or column chromographic separations and preceded the explosion in knowledge of intermediary metabolism and endocrine functions. Almost no metabolic studies have been reported in experimentally induced human infection.

In setting up our ward and laboratory facilities and in beginning our studies on the influence of acute infection on host metabolism, we have attempted, initially, to secure good baseline data to survey and correlate numerous areas of metabolic interest. We have superimposed an elaborate metabolic balance technique upon 16 of the volunteer studies conducted during the past year and in addition we have been able to obtain urinary steroid analyses in 32 other volunteer studies. To permit you to judge the data obtained in these studies, it is necessary initially to spend a brief period in describing the design and techniques of the balance methods we employ.

METHODS AND MATERIALS

Figure 1 depicts the general design of the metabolic balance studies. With exposure planned for day 0, the volunteers are admitted 13 days earlier and begin on their diets. Urine and stool collections begin the following morning. The first 4 days on the ward are devoted to dietary equilibration and to familiarization of the men with the metabolic methods employed. Urine specimens were collected every 24 hr and stool specimens in 3 day increments demarcated by colored-dye markers given at the times indicated by an X. Blood is sampled as indicated throughout the 9 day control period and during the period of incubation, illness, and convalescence. Control urine for 3 days is analyzed for adrenal steroid content with daily analyses being obtained thereafter.

* U. S. Army Medical Unit, Fort Detrick, Maryland.



X = START MARKER

B = BLOOD

FIGURE 1. METABOLIC BALANCE STUDIES — GENERAL DESIGN.

12

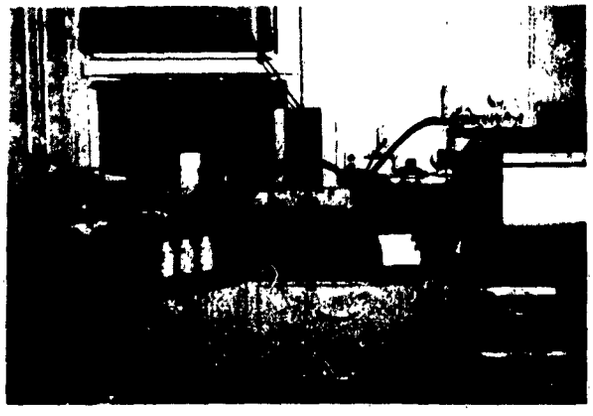


Figure 2. Preparing the metabolic diet.

Figures 3 & 4. Ward activities.

Figure 5. Aliquoting metabolic samples.

Figure 6. Automated analysis equipment.

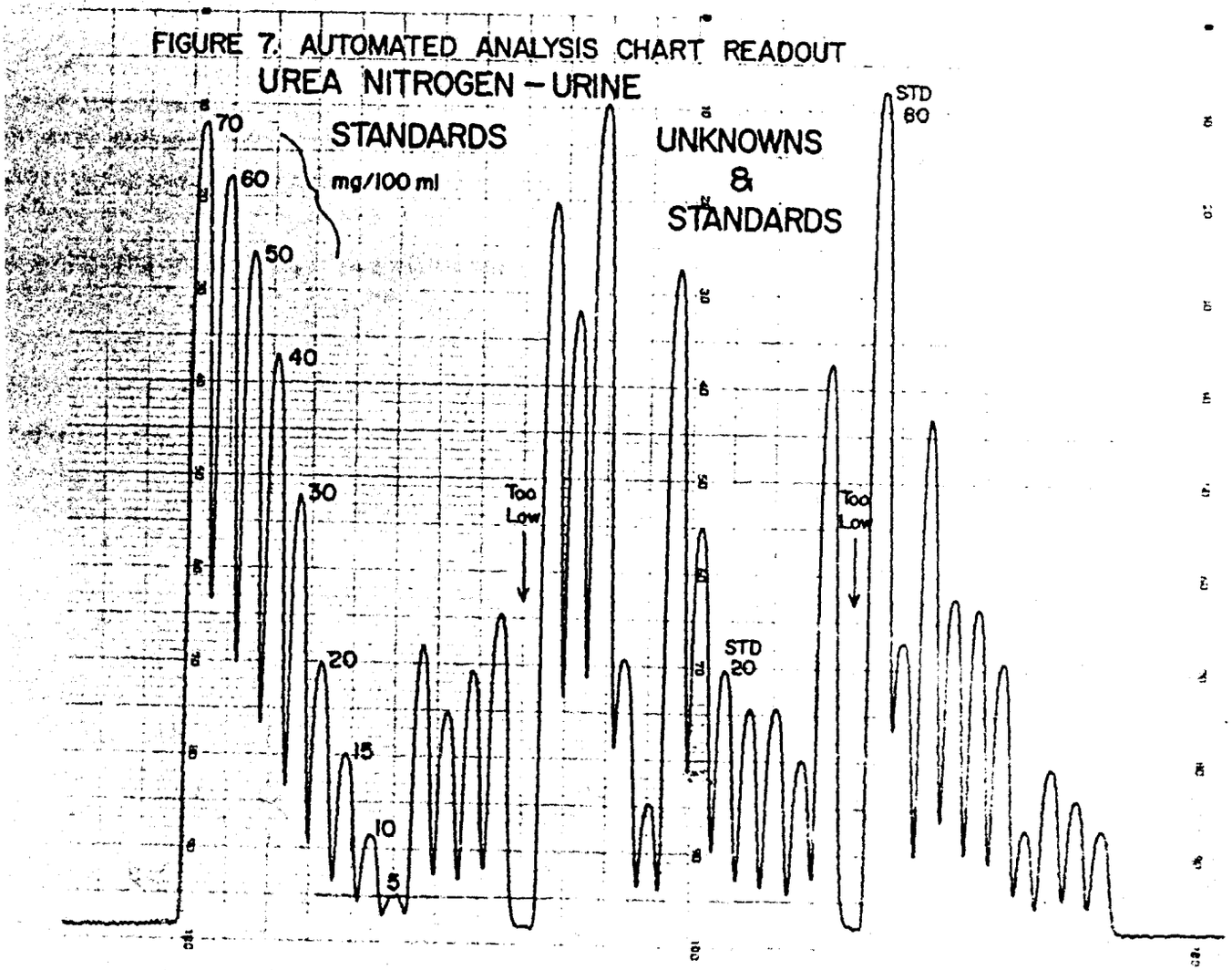


FIGURE 7. AUTOMATED ANALYSIS CHART READOUT
UREA NITROGEN – URINE

A constant liquid formula diet of nonanimal origin was designed by Major Florence Berger, Metabolic Dietitian, Walter Reed Army Institute of Research. This was prepared each day and served by weight according to weight and body configuration of the individual subjects. In addition, 5 varieties of fruit were provided each day. These were obtained from single-lot sources prior to study, drained, weighed into Dixie cups, and held frozen until the day of use. The diet contains normal protein, fat, carbohydrate ratios with adequate minerals and electrolytes. Figure 2 shows the preparation of fruit in Dixie cups in our metabolic kitchen. All procedures involved in diet preparation, serving, and in stool and urine collections were devised to combine simplicity with accuracy so that it was possible to develop adequate corpsmen and technician skills in a very brief period of time. To avoid boredom during the long control and postcontrol periods, various amusements were provided for the patients, such as those shown in Figures 3 and 4.

Stool and urine samples and diets were analyzed for total nitrogen, sodium, potassium, calcium, phosphate, and magnesium while urinary samples were additionally studied for chloride, urea, ammonium, creatine, uric acid, and amino acid content. Stool, urine, and dietary samples were quantitated and aliquoted on a weight basis (Figure 5). All of the chemical procedures were performed using automated equipment such as shown in Figure 6. These individual pieces of equipment, manufactured by Technicon, could be modified and adapted for research precision. They have in fact made a study of this sort feasible, since over 2,000 chemical determinations are required for each individual included in a study such as this. Figure 7 shows an example of the urinary urea nitrogen readout. We begin each tray with sufficient standards to bracket all the unknowns. Thereafter, unknowns and known samples are included, and the results are determined by comparing the heights of color development of the unknown samples with the standards which bracket them. We can run determinations of rates varying from 20 to 80/hr. In doing these analyses, all samples were held frozen to the end of a study at which time all samples of a given individual were run consecutively. Analyses were done in replicate and rechecked if these did not agree.

The urinary steroid analyses are being performed in the steroid laboratories of the Department of Metabolism at WRAIR under the direction of Mr. Joseph Bruton. In addition to general analyses of 17-hydroxycorticoid steroids, 17-ketosteroids, and pregnanetriol fractions, the individual metabolites which comprise these latter 2 fractions are being studied in greater detail by separating them individually, using column and gas chromotography.

RESULTS: ADRENOCORTICAL RESPONSES

The results of our studies may be subdivided into the adrenocortical portions and the balance portions. Figure 8 depicts the adrenocortical response in 12 volunteers who developed acute tularemia following exposure to an aerosol of Pasteurella tularensis. At the top is shown the mean

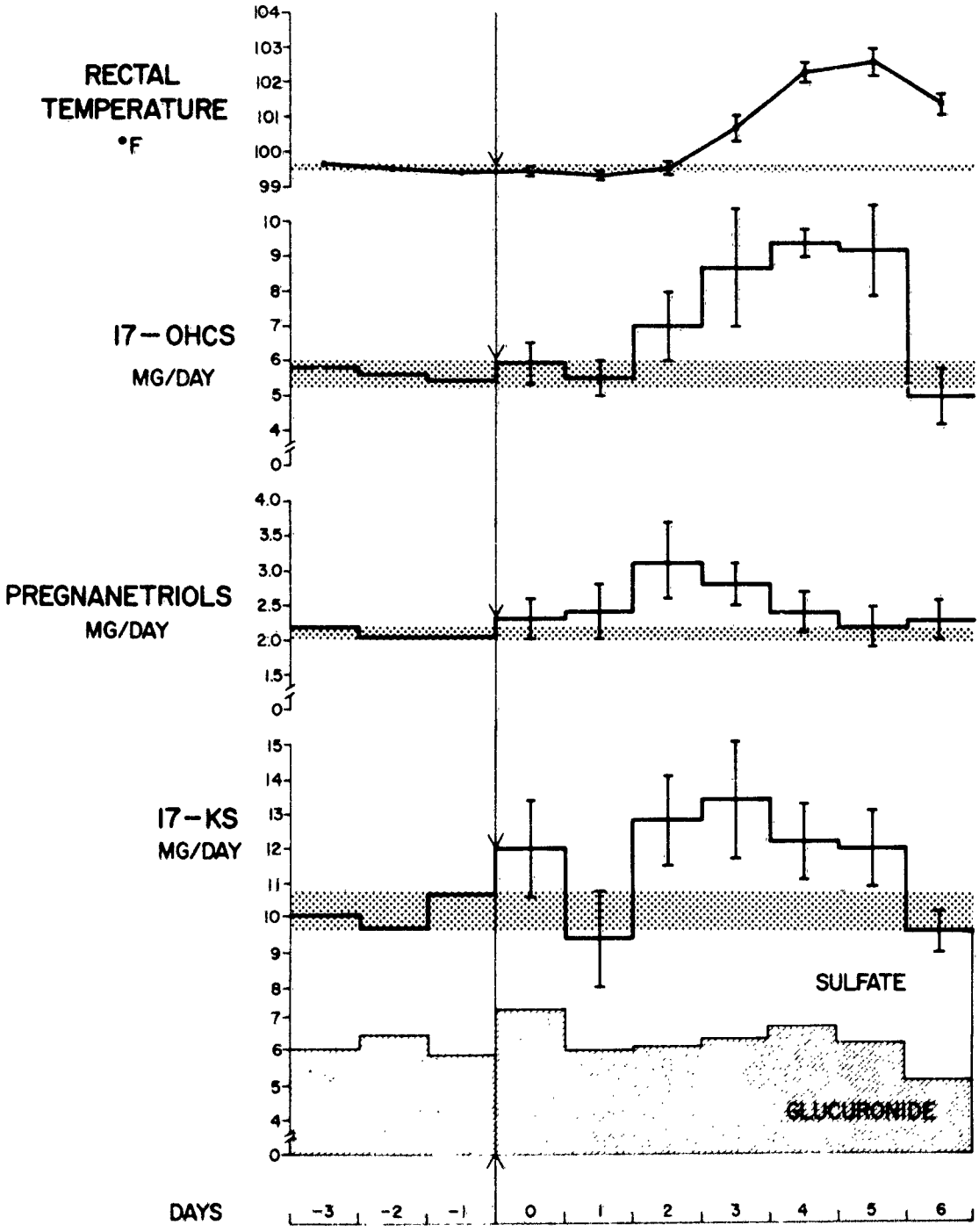


FIGURE 8. URINARY CORTICOSTEROID EXCRETION IN 12 SUBJECTS WITH INDUCED ACUTE TULAREMIA.

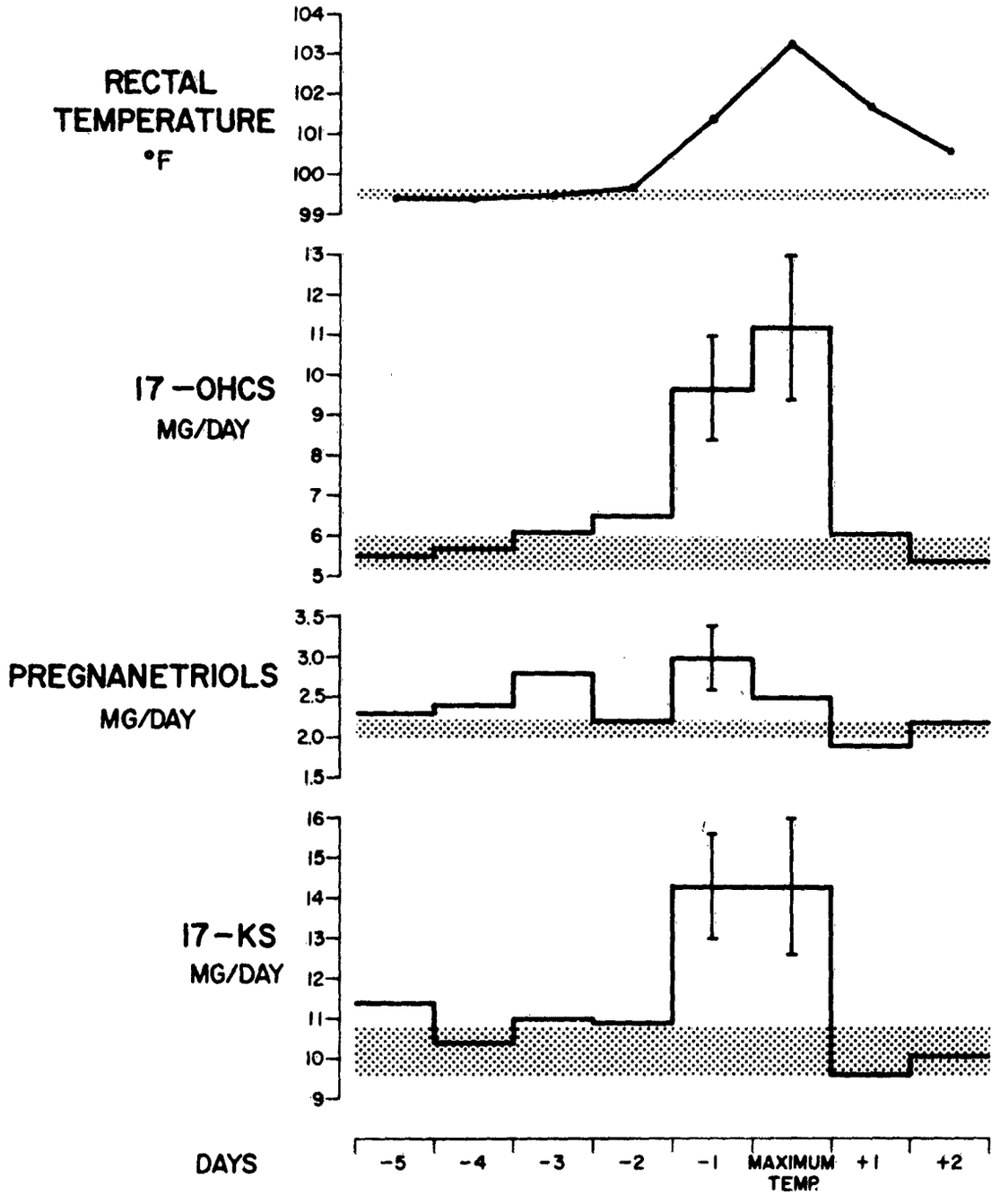


FIGURE 9. URINARY CORTICOSTEROIDS IN 12 SUBJECTS WITH ACUTE TULAREMIA.

maximum rectal temperature for this group. The shaded horizontal areas represent the average, plus or minus one standard error of the control data. Coincident with fever, there was an increase in the 17-hydroxy- and 17-keto-steroid metabolites in the urine. Pregnanetriol excretion showed a tendency to rise early in fever but returned to baseline as fever and symptoms became maximal. This same data is replotted in Figure 9 with the day of maximum fever of each patient being placed at day 0 and the results of the days which preceded and followed it shown as minus or plus days. This gives us a somewhat different view of the adrenocortical response showing a gradual progressive rise in hydroxycorticoid excretion coincident with fever and an abrupt return to normal with the institution of therapy, even though fever and symptoms persisted for several additional days. Ketosteroids showed a sizeable increase only at the height of fever and these too returned to normal abruptly with institution of therapy. This arrangement of the data lessens the apparent significance of the pregnanetriol rise early in fever. Figure 10 shows data in a group of 8 immunized subjects. Here are shown a lesser degree of fever and virtually no adrenocortical response. When this data was replotted to separate the 4 men in this group who became symptomatic and febrile from 4 who did not, as shown in Figure 11, there appeared to be no appreciable difference in the adrenocortical response of both groups. Similarly, nonimmunized subjects exposed to attenuated organisms, sham exposed individuals, and individuals with acute tularemia who were later re-exposed, showed no significant or consistent change in their pattern of adrenal steroid excretions even though some subjects showed mild symptoms and fever during the period of urine collections.

NITROGEN STUDIES

The metabolic balance studies included balances for nitrogen, sodium potassium, calcium, phosphate, and magnesium. Figure 12 depicts the nitrogen balances, shown as the averages of 9 subjects with acute tularemia. To the left are shown data plotted chronologically, and to the right are shown the data following exposure replotted as done with the steroids, according to the time of maximum fever. Temperature results are shown at the top of this figure and the ones that follow. Below this is plotted the nitrogen balance in the fashion popularized by Albright and Reifenstein. Changes in the urinary excretion of nitrogen are plotted next; the horizontal shaded area representing the daily excretion during the control period plotted as the mean plus or minus one standard error. At the bottom of the slides are shown the cumulative balance expressed as a deviation from the control average. This last arrangement of the data arbitrarily assumes that the subjects are in balance at the end of the control period; nitrogen accumulation is represented by an upward deflection and loss from the body as a downward deflection. It can be seen that the nitrogen balance became negative shortly after the height of fever had been reached. Negative balance was due to both a reduction of dietary intake of protein associated with the anorexia of the acute illness, and in addition to increased losses of urinary nitrogen. When this is plotted cumulatively it can be seen that an abrupt loss developed at the height of illness and

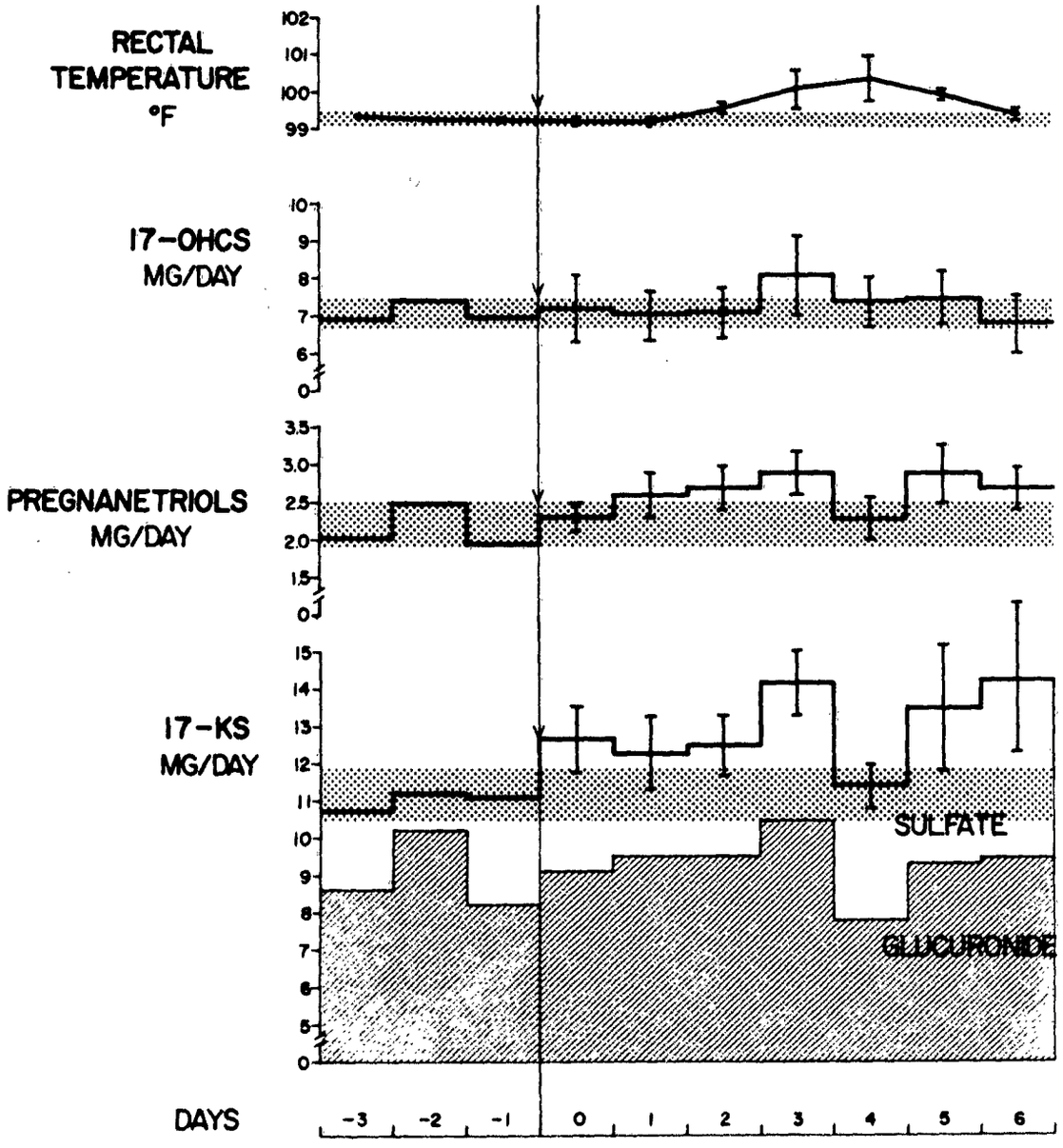


FIGURE 10. URINARY CORTICOSTEROID EXCRETION IN 8 IMMUNIZED SUBJECTS EXPOSED TO P. TULARENSIS.

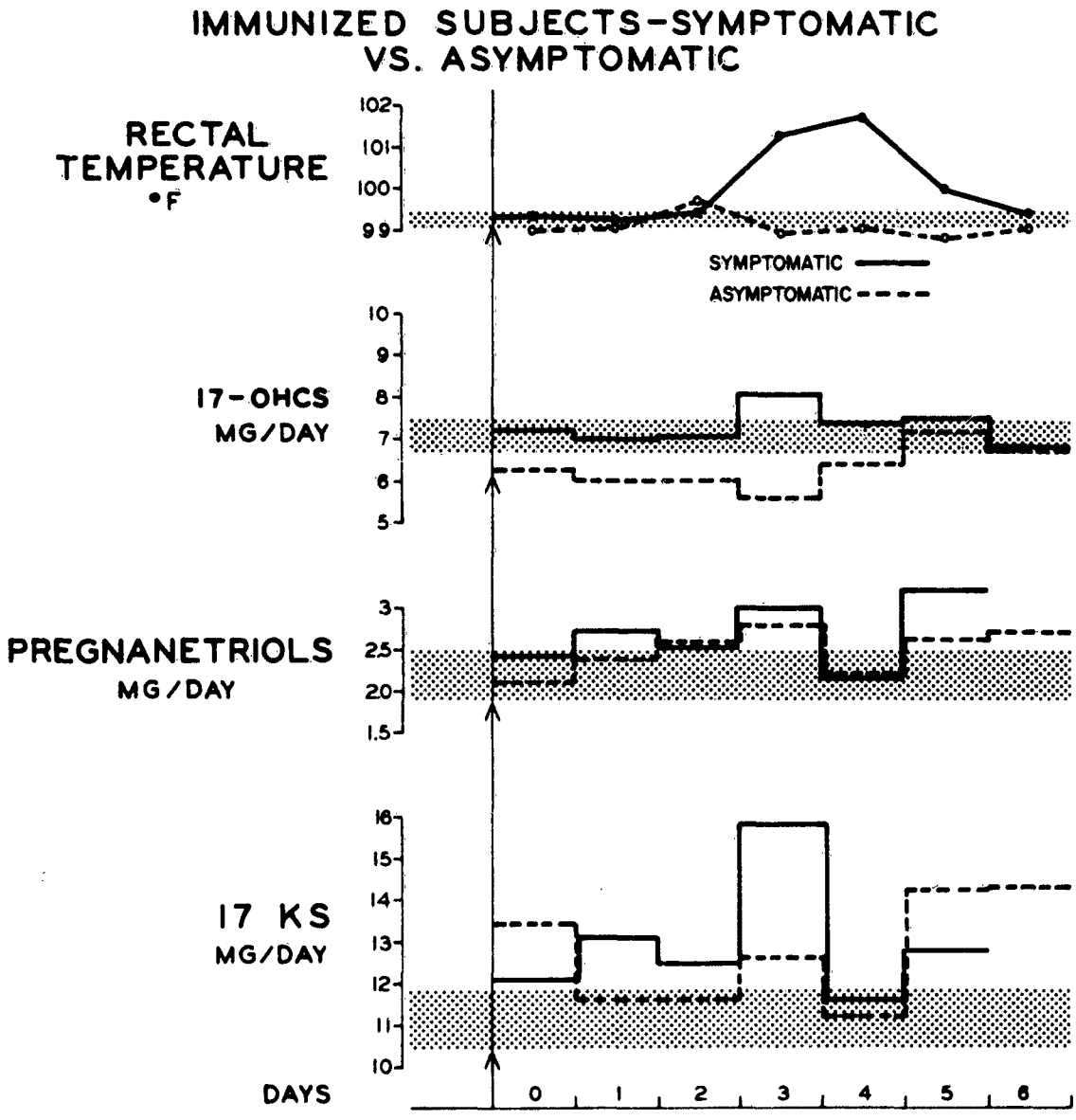


FIGURE 11. URINARY CORTICOSTEROID EXCRETION IN IMMUNIZED SUBJECTS— 4 SYMPTOMATIC AND 4 ASYMPTOMATIC.

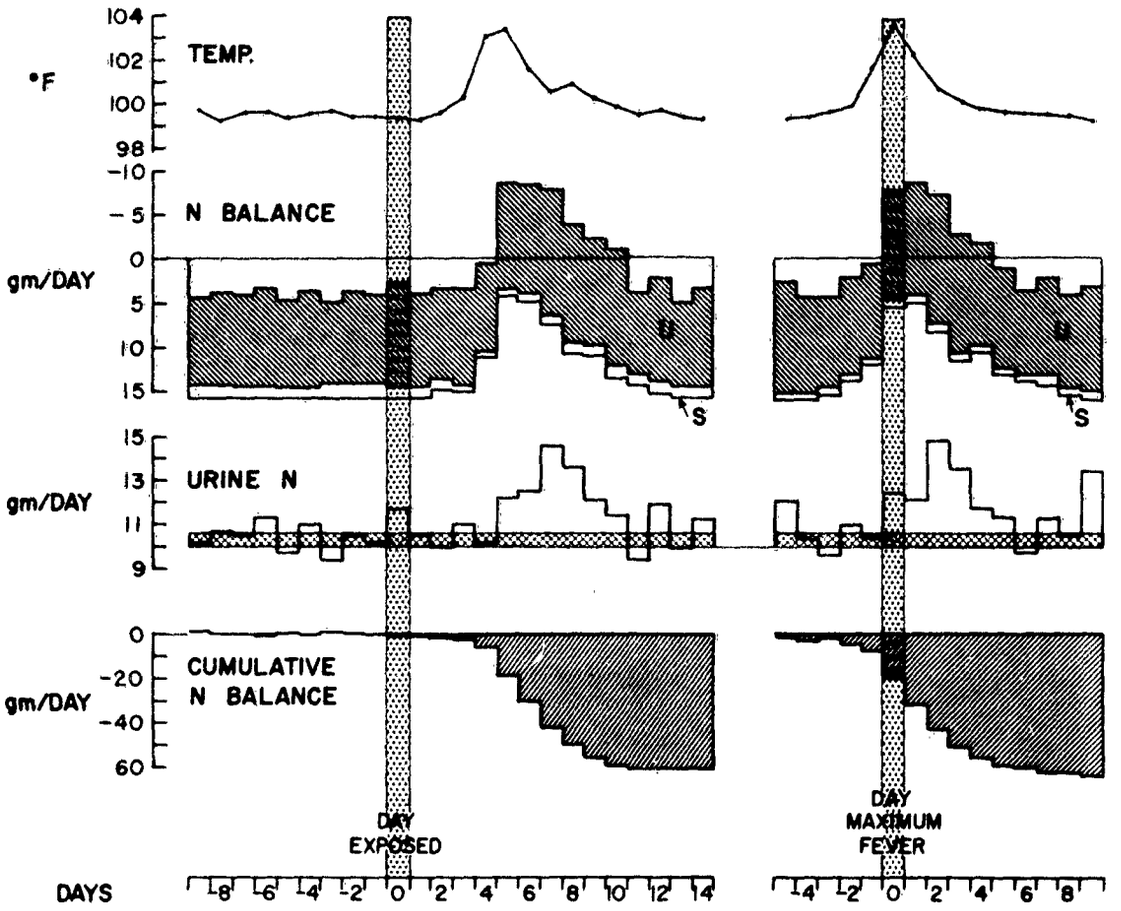


FIGURE 12. NITROGEN BALANCES IN 9 SUBJECTS WITH ACUTE TULAREMIA.

extended at a slower rate into convalescence, persisting to the end of the study. Figure 13 shows the partition of losses of nitrogen in the urine. All data here are superimposed upon horizontal shaded bars which depict the control mean, plus or minus one standard error. As noted by earlier investigators, an increased loss of urinary creatinine closely paralleled the febrile response. Increased losses of urinary urea, ammonia, and amino acids became maximal later during the lysis of fever; during this period a slight cut-back in uric acid excretion was observed, possibly due to diversion of nitrogen away from uric acid synthesis. Note that the rise in amino acid excretion was followed by a prolonged fall in their urinary losses which possibly indicates their utilization in rebuilding lost body proteins.

ELECTROLYTE STUDIES

Electrolyte changes are shown in the Figure 14; sodium and potassium balances are shown above. A negative Na balance developed shortly after fever and symptoms were maximal due both to a decrease in dietary intake and an increased loss of urinary sodium. Na losses were, however, corrected abruptly; in some patients urinary Na became almost undetectable. Note that this change in the renal handling of Na occurred several days following the time of reversion of adrenocortical overactivity to baseline levels. The negative K balance began coincident with that of Na loss, but persisted for a much longer period of time. This was due again both to a decreased intake and to slightly increased urinary losses. The urinary Cl changes are depicted next and show essentially the same pattern of urinary loss as did Na. Below are shown the cumulative balances for Na and K. Note that these differ, in that the abrupt losses of Na were reversed fairly promptly while progressive losses of K persisted to some degree throughout convalescence.

PHOSPHATE STUDIES

In Figure 15 phosphate balance is shown. Again, negative balances resulted from decreased dietary intake and increased urinary losses. The plot of the cumulative balance shown at the bottom again portrays a period of abrupt loss coincident with acute illness, with losses diminishing but persisting throughout observed convalescence.

MAGNESIUM STUDIES

In Figure 16 magnesium balances and cumulative loss are seen to follow an identical pattern to that of the other intracellular components - N, K, and PO_4, with abrupt cumulative losses gradually slowing but still persisting throughout the observed period of convalescence.

CALCIUM STUDIES

Calcium balances are shown in Figure 17. While negative balances did occur during acute illness, Ca, like Na, and unlike the major intracellular elements, showed a prompt reversal of the slight losses incurred.

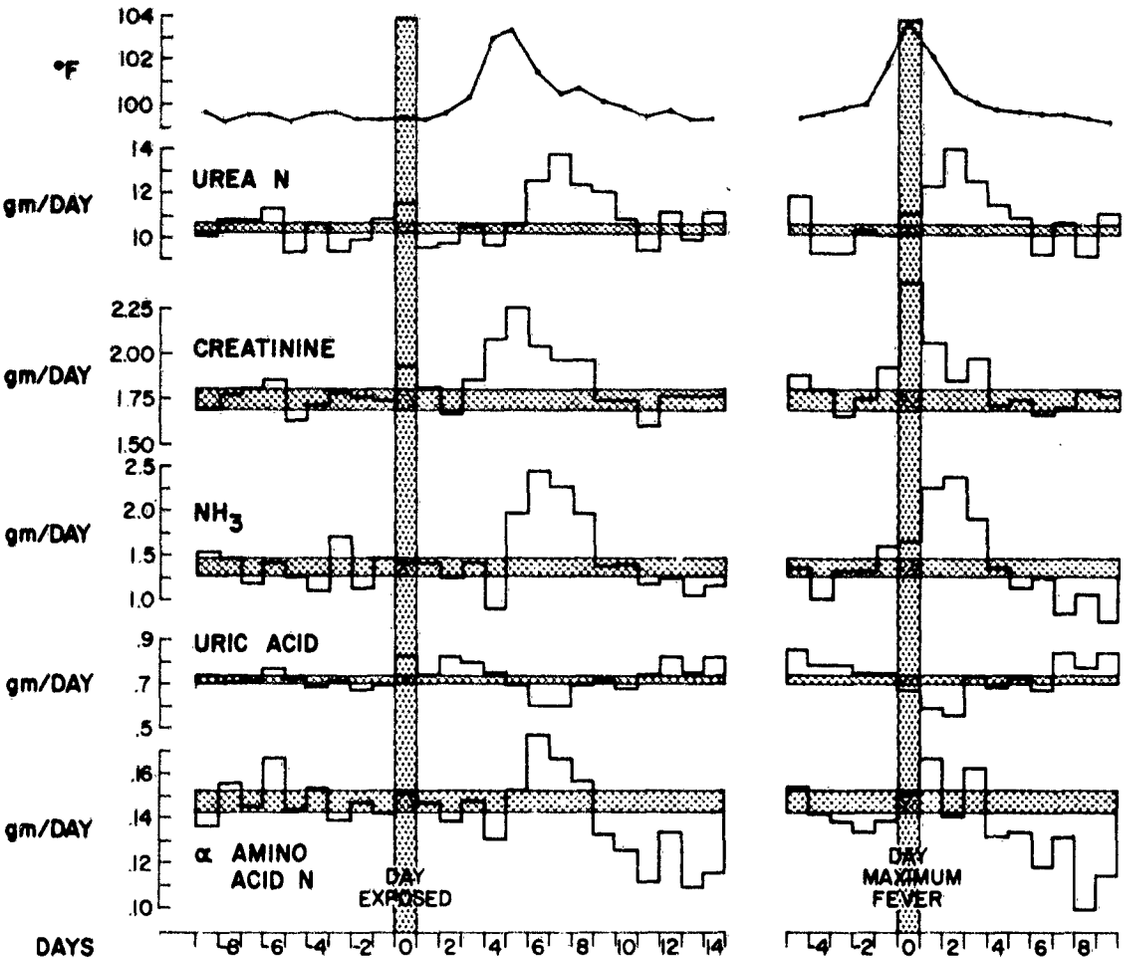


FIGURE 13. URINARY NITROGEN COMPONENTS IN 9 SUBJECTS WITH ACUTE TULAREMIA.

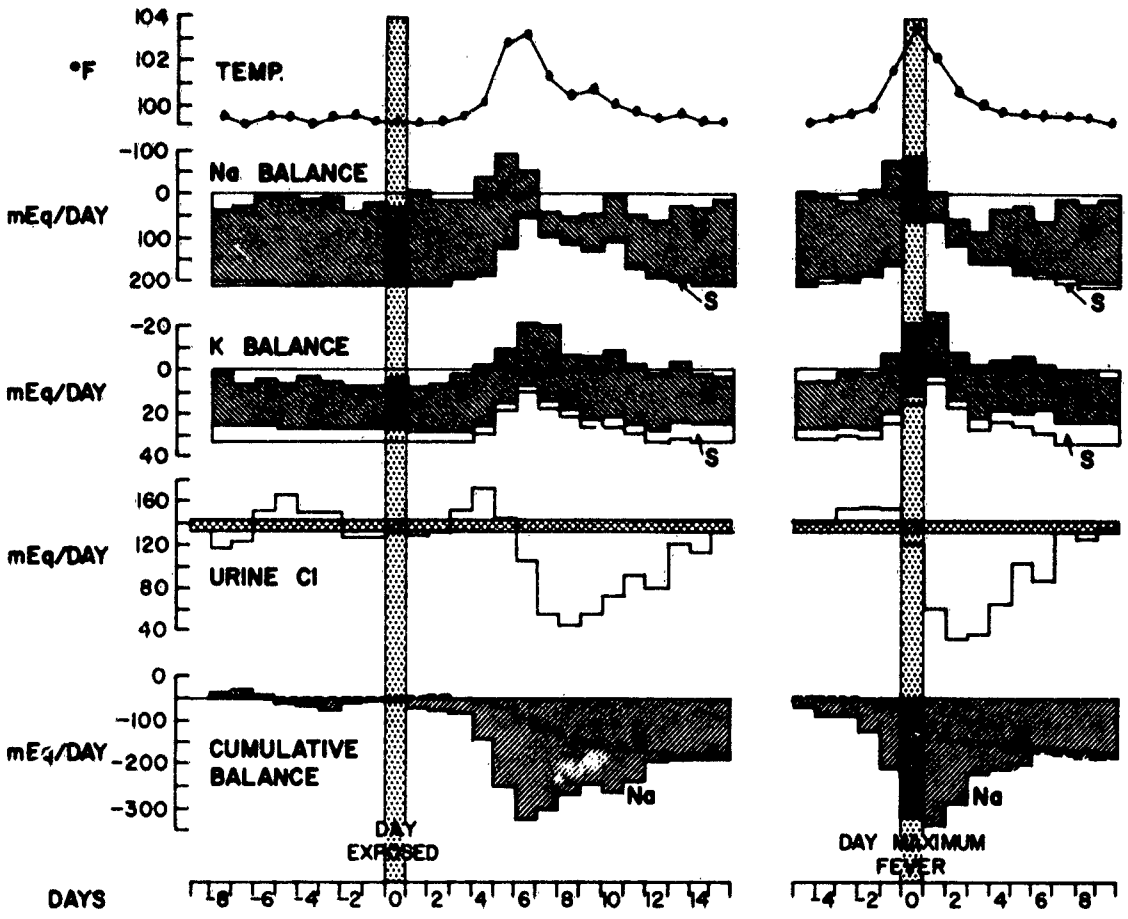


FIGURE 14. ELECTROLYTE BALANCES IN 9 SUBJECTS WITH ACUTE TULAREMIA.

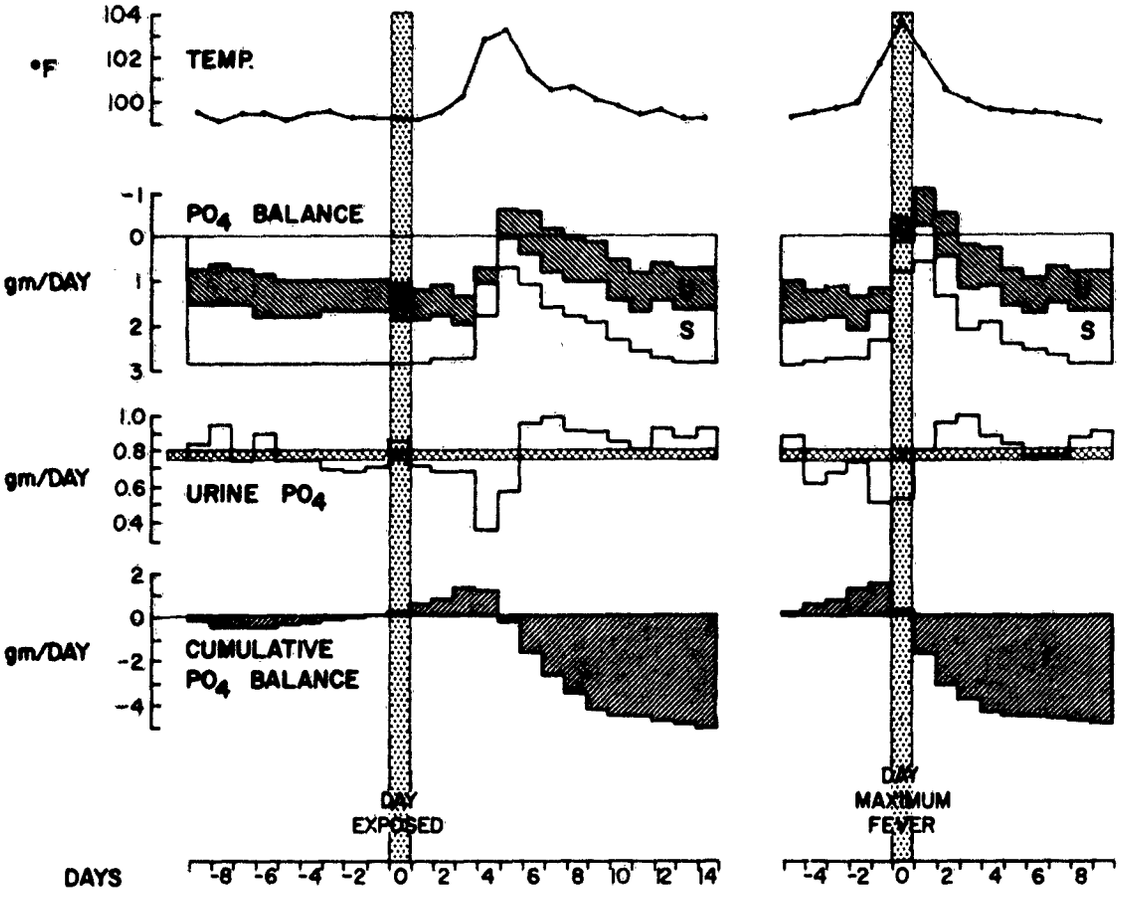


FIGURE 15. PHOSPHATE BALANCE IN 9 SUBJECTS WITH ACUTE TULAREMIA.

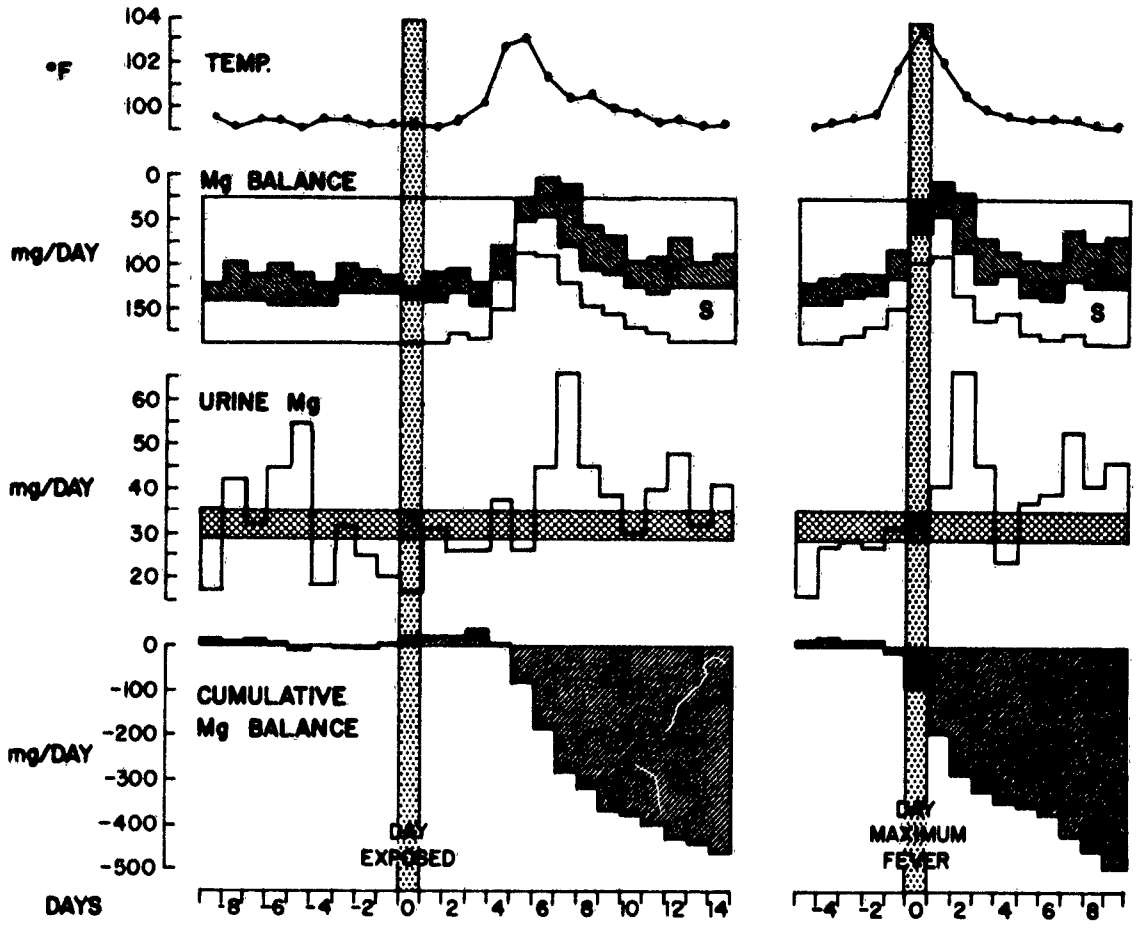


FIGURE 16. MAGNESIUM BALANCES IN 9 SUBJECTS WITH ACUTE TULAREMIA.

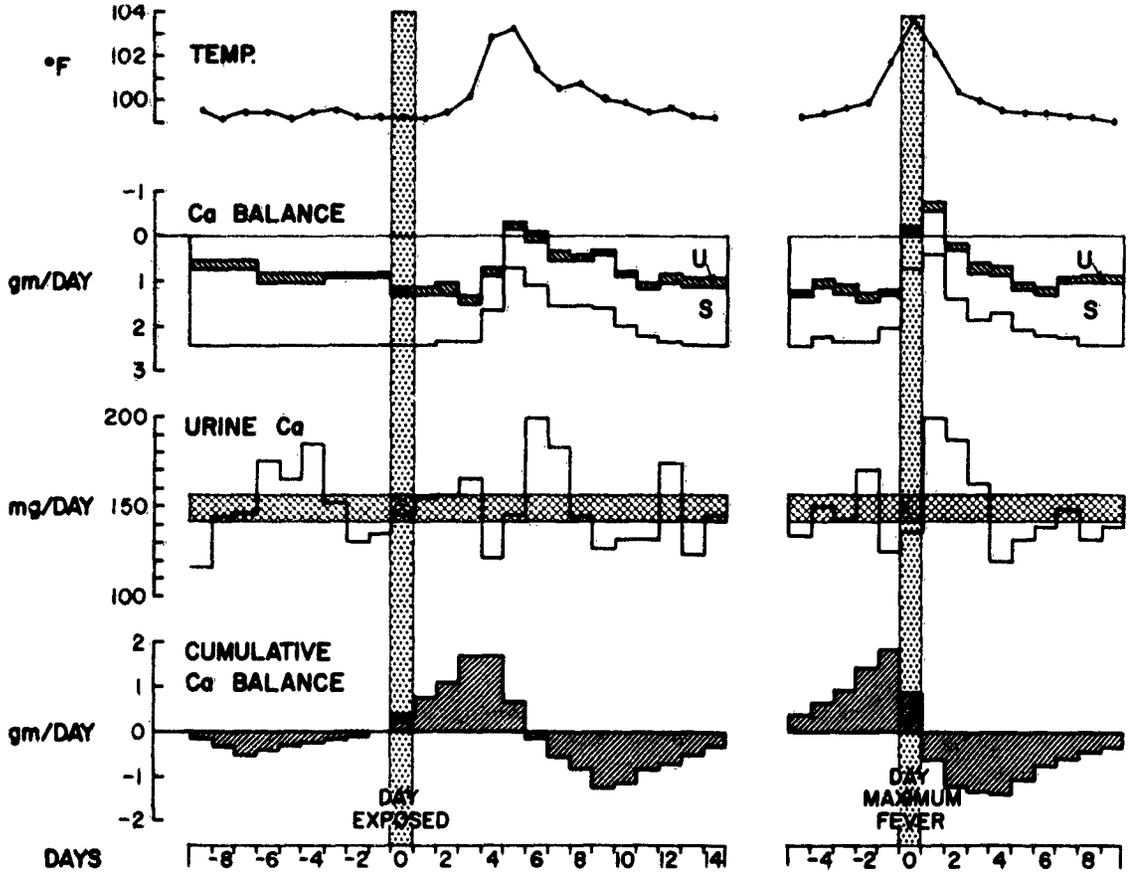


FIGURE 17. CALCIUM BALANCE IN 9 SUBJECTS WITH ACUTE TULAREMIA.

BLOOD CHEMISTRIES

In these acutely ill subjects, only minimal changes were noted in nitrogenous compounds (Figure 18), or in serum minerals (Figure 19). A significant but slight fall in both Na (Figure 20) and Cl was observed, confirming the work of others.

CONTROL STUDIES

No significant changes in balance were observed in 4 immunized subjects who did not become ill after exposure. Nonimmunized subjects who showed only mild symptoms and slight fever showed minimal deviations in metabolic balances. These were of a similar nature but were lesser in degree than those of the acutely ill group.

DISCUSSION

In considering the data presented, several things seem clear. First, acute tularemia, treated promptly in these healthy young males was associated with increased adrenocortical activity; this was modest in degree, similar to mild Cushings disease. An increased adrenocortical response was not touched off by mild disease. Second, the mainly intracellular elements differed from those mainly extracellular. Na, Cl, and Ca showed early losses which were corrected fairly promptly, while the loss of intracellular components was protracted. Third, the timing of changes in balance was not coincident with that of adrenocortical response. It remains a likely possibility that mineralocorticoid stimulation occurred in conjunction with the renal retention of Na; accordingly we plan to measure these urines for aldosterone content. Fourth, alteration in intermediary pathways of N metabolism was evidenced by the noncoincident changes in excretion of various metabolites.

Naturally, many questions remain unanswered and many variables unexplored; fat and carbohydrate metabolism and other endocrine changes have yet to be studied. Variables of dietary content, nutritional status, muscular condition, fatigue, and the like must eventually be considered. Additional studies are now under way to determine the adrenal and metabolic effects of fever per se, as induced by external heat and by bacterial endotoxins. Future studies are planned to study these same responses in acute illnesses other than tularemia. In addition, studies in monkeys are in progress in which a constant daily gastric gavage feeding will prevent changes in metabolic balance attributable to altered dietary intake as seen during acute human disease. In addition, data in the monkeys will extend the metabolic observations to untreated disease terminating in death.

SUMMARY

In summary, data on the adrenocortical responses and metabolic balances of N, electrolytes, and minerals have been reported in groups of volunteer subjects exposed to an aerosol of P. tularensis. The results

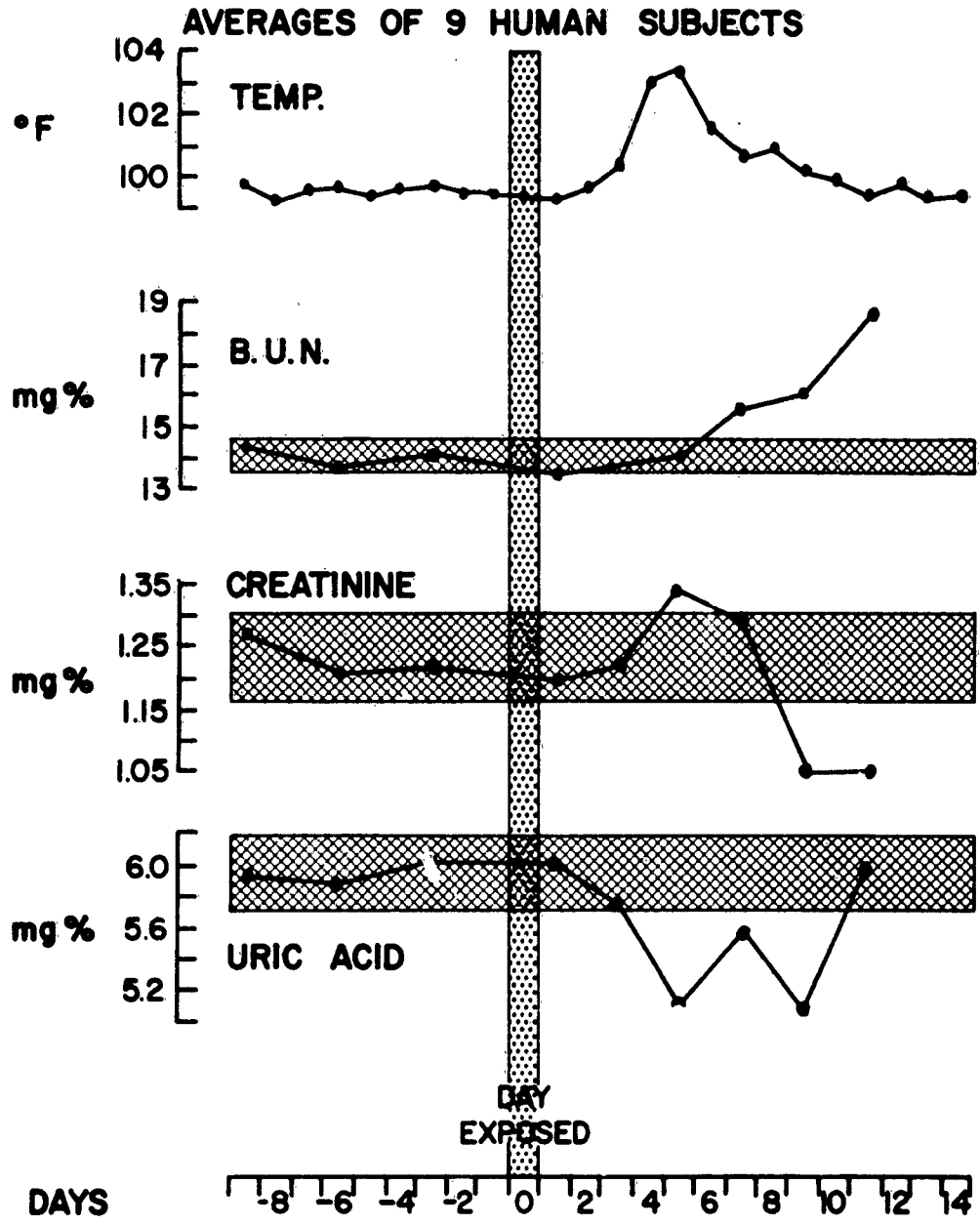


FIGURE 18. SERUM NITROGENOUS COMPOUNDS IN 9 SUBJECTS WITH ACUTE TULAREMIA.

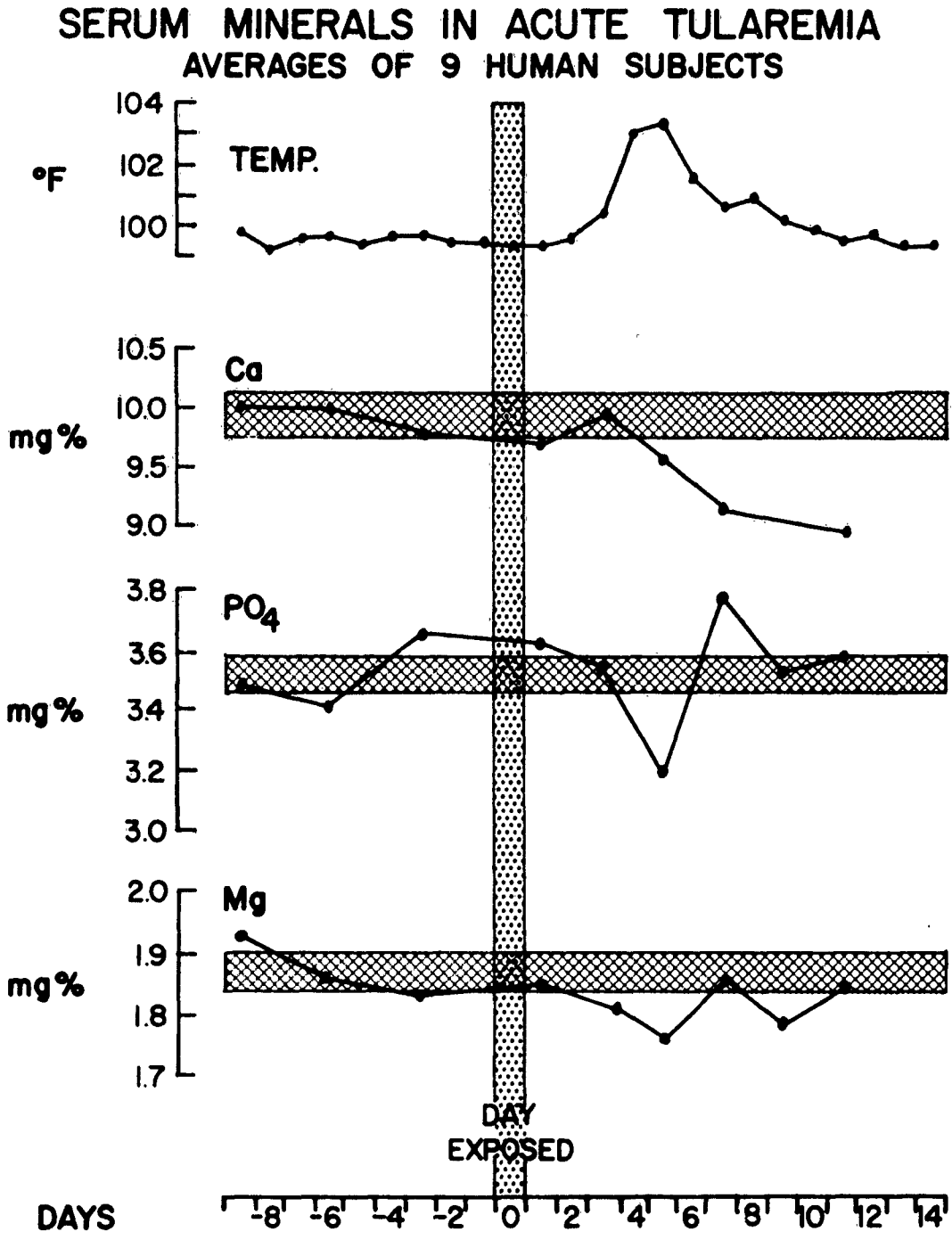


FIGURE 19. SERUM MINERALS IN 9 SUBJECTS WITH ACUTE TULAREMIA.

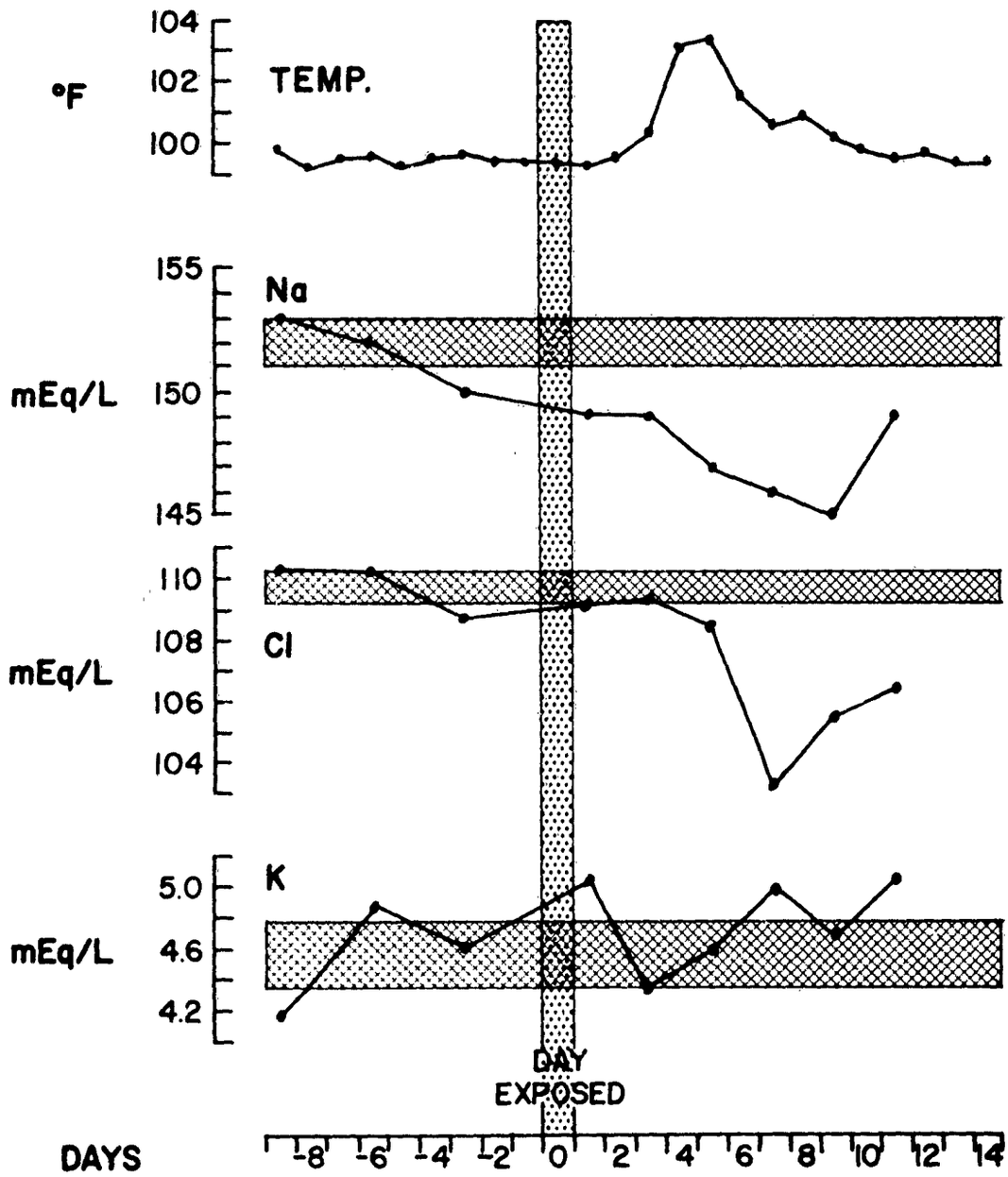


FIGURE 20. SERUM ELECTROLYTES IN 9 SUBJECTS WITH ACUTE TULAREMIA.

show an increase of adrenocortical activity coincident with a developing fever of patients who became acutely ill with tularemia but little or no change of significance in the other groups studied. Balance data revealed an essentially similar pattern for the major intracellular elements -- N, K, PO_4, and Mg, with abrupt loss developing slightly after the height of illness. While this loss diminished in degree, it persisted long into convalescence. On the other hand, losses of Na, Cl and Ca occurred abruptly and appeared to be reversed rapidly.

It must be noted that the major changes in balance occurred after the adrenocortical response to acute illness had subsided. Thus, balance changes appeared to be clearly differentiated from adrenocortical response by this separation of time.

In concluding this presentation, I would be severely remiss if I did not express my appreciation to the volunteer subjects whose cooperation and fortitude were necessary to make a study such as this possible. I also wish to thank the various corpsmen and technicians who comprise our Metabolic Research Team.

FLUORESCENT ANTIBODY TECHNIQUE

Robert F. Jaeger, B.A.*

The use of the fluorescent antibody procedure as a specific, rapid and convenient laboratory diagnostic technique for Venezuelan equine encephalomyelitis (VEE) from serum samples of man is indicated.

Easterday and Jaeger[1/] showed that Rift Valley fever virus could be rapidly identified using a tissue culture-fluorescent antibody system. They demonstrated that time of detection was dose-dependent, that is, that as the virus to cell multiplicity increased the time of positive findings decreased.

The present report is based on this finding. However, marked modifications in the materials and methods greatly facilitated the handling of the material being examined.

Second passage guinea pig heart cells were suspended in Eagle's basal medium + penicillin-streptomycin (100 units/ml) and 20% horse serum at a concentration of 5×10^5 to 1×10^6 tissue cells/ml. From this suspension 1.8 ml was placed into Falcon 35 x 10 mm plastic tissue culture Petri dishes and incubated at 37 C for 18-24 hr. Without removing the growth medium, 0.2 ml of the serum was added directly to the medium in each culture plate.

The serum was obtained from 2 individuals who had been infected with 5 and 18 mouse intraperitoneal lethal $doses_{50}$ ($MIPLD_{50}$) respectively of Trinidad strain VEE. Clotted blood samples were collected daily and the serum treated in the usual manner.

If the test were to be delayed for several hours or days the serum samples were stored at -60 C until used.

At various time intervals replicate plates for specific staining and additional control plates were removed from the incubator, washed in sterile balanced salt solution, fixed in 10% formalin (pH adjusted to 7.3), stained with appropriate conjugate for 30 minutes at 37 C, washed for 15 minutes in phosphate buffered saline (PBS), pH 7.2; excess PBS was removed and a coverslip applied using a PBS-glycerin solution at pH 7.2.

The following conjugates were used either as specific or control stains. Burro anti-VEE, normal burro, rooster anti-*Pasteurella tularensis*, monkey anti-Rift Valley fever and monkey anti-yellow fever. All conjugates were prepared in a similar manner, first having been fractionated by the methanol method of Dubert *et al*[2/] as modified by our laboratory.

Results indicated that in case No. 1 (C.D.), it was possible to identify specifically the VEE virus in the tissue culture cells in as short a time as

* U. S. Army Medical Unit, Fort Detrick, Maryland.

4 hr postinfection. This was at a viremia level of $10^{8.5}$ logs as determined by mouse intracerebral titration ($MICLD_{50}$). Case No. 2 (A.M.) where the viremia level was considerably lower ($10^{5.5}$ logs) 13 hr incubation was required. These times are markedly short when compared to the time required for mouse neutralization, hemagglutination inhibition and complement fixation tests.

The above dose and times are comparable to those reported by Easterday and Jaeger[1/] for the recovery of Rift Valley fever virus.

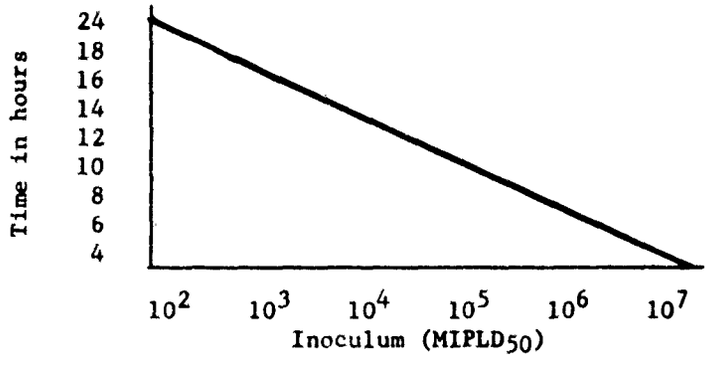
Subsequent to obtaining these data, Jaeger and Wooding reported on the variation in recovery of VEE virus at a given dose in 5 different cell lines. Guinea pig heart cells, although they appear to be more readily infected by VEE virus, must be used on 2nd passage. This fact could cause their use to be restricted. It has been shown that Chang human liver cells and HeLa (Gey) cells using 20% horse serum in Eagle's basal medium give comparable results.

Viremia levels as low as 10^2 $MICLD_{50}$ in the case of VEE and as low as 10^2 $MIPLD_{50}$ for Rift Valley fever can be specifically demonstrated by the tissue culture-fluorescent antibody procedure within 24 hr following initial infection of the tissue culture system.

The above reported work along with presently incomplete studies would indicate that a rapid tissue culture-fluorescent antibody virus titration method may be developed.

Figure 1 is a composite of several experiments. The times of detection of specific fluorescence will vary with the tissue culture cell line employed. The graph does indicate the rapidity of obtaining results such a titration technique would afford.

FIGURE 1. RELATION OF VIRUS INOCULUM TO IDENTIFICATION TIME IN TISSUE CULTURE



In respect to modification in materials and methods previously mentioned, the following techniques or changes in standard procedures have been found to improve materially the staining quality, increase the ease of handling of tissue culture cell lines, and decrease the time required for cell line growth.

INCREASED GROWTH OF TISSUE CULTURE CELLS

Having sufficient tissue culture cells available when needed has frequently been a determining factor governing the number of samplings and how frequently replicate tests can be conducted.

By making the following alteration in the growth medium used in conjunction with Chang human liver cells, several days can be eliminated between seeding flasks and harvesting of cells.

Using Eagle's basal medium, 20% horse serum is added. Combined penicillin-streptomycin may be added if desired. If utilized, the antibiotics are added at a concentration of 100 units/ml.

Using NaOH, the pH of the medium is adjusted to 7.8. Incubation is carried out at 35 C.

The seed cell concentration is 1 x 10^6/ml; 1 ml of this tissue cell suspension is added to an 8-oz prescription flask containing 15 ml of medium.

Within 3-4 hr after placing flasks in the incubator, visual adherence of the cells to the glass can be seen. Sufficient growth for harvesting has occurred in 48-72 hr.

ENHANCING FLUORESCENCE

Pital and Janowitz[3/] have reported that by markedly altering the pH of the final wash and glycerin mounting fluid, intensity of fluorescence will be increased. Along with the increase in pH a shift to sodium carbonate-sodium bicarbonate buffer was recommended. It is their opinion that at pH 9.0 the sodium carbonate-sodium bicarbonate buffer is more stable than phosphate buffer at a similar pH.

Table I shows the effect of pH and buffer on the intensity of fluorescence and the maximum usable dilution of freeze-dried avian anti-P. tularensis SCHU-S4 conjugate. At the time of freeze-drying in March 1961, this particular conjugate had a working dilution (that is 4+ staining intensity of a 36-hr culture "GCBA" of P. tularensis) of 1.128. A gradual diminution of staining intensity and a resulting lowering of the working dilution had been noted over a 2½ year period when this conjugate was reconstituted according to our original instructions.

As indicated in the table the use of sodium carbonate-sodium bicarbonate buffer at pH 9.6 has restored the intensity of fluorescence to near its original intensity.

TABLE I. EFFECT OF pH AND BUFFER ON STAINING INTENSITY OF AVIAN ANTI-P. TULARENSIS CONJUGATE[a]

		INTENSITY AT DILUTIONS			
		1:16	1:32	1:64	1:128
DH_2O[b]	pH 7.0	2+	1+	±	±
PBS	pH 7.15	3+	1+	±	±
Sodium carbonate-sodium bicarbonate	pH 9.6	4+	4+	4+	2+

a. Conjugate freeze-dried March 1961.

b. Distilled water.

It is interesting to conjecture as to what the maximum dilution giving 4+ staining would have been had this conjugate originally been diluted with sodium carbonate-sodium bicarbonate buffer in the pH 9.0 range.

USE OF SMALL PLASTIC PETRI DISHES

A third modification has been the development of a new technique for the preparation and fluorescent staining and examination of virus-infected tissue cultures. The use of Falcon plastic tissue culture petri dishes (35 x 10 mm) permits more rapid and precise processing of specimens, reduces amounts of inoculum required and promotes safety in handling infectious materials. It has been determined that the handling of the specimens when compared to the cover slip technique or half cover slip in Leighton tube technique has been effectively reduced as much as 40%. The labeling and specimen identification is easier and more exact. No longer is one plagued by having a cover slip placed upside down on the slide. Specimen loss due to breakage is effectively eliminated. Since the plastic dishes are pre-cleaned and sterile, cleaning procedures are unnecessary. The ratio of virus to cells could be altered in each dish to suit experimental variations.

In addition, the "safety factor" needed when studying highly pathogenic viruses is much greater, due principally to the fact that there is far less opportunity for spillage.

Finally each petri dish acts as its own "moist chamber" during the staining period.

Two disadvantages are: first, the initial cost of the plastic tissue culture petri dish is considerably higher than that of the glass cover slip, but this is offset to a great extent by the lack of preparation needed, the

reduced handling time and the added safety factor; second, due to the slightly larger area of the petri dish, a greater amount of conjugate is required to cover the area.

STAPHYLOCOCCAL ENTEROTOXIN DETECTION

In view of the current interest in staphylococcal enterotoxin, protocols were prepared and tests done to determine the feasibility of employing the fluorescent antibody technique in the detection of low concentrations of staphylococcal enterotoxin.

Employing a checkerboard titration method, it was possible to detect the presence of PB-9-B enterotoxin at a concentration of 0.01 µg in a 0.01 ml spot. Concentrations of the enterotoxin below this level have not as yet been investigated.

Some negative reactions in the areas of great antigen excess have been encountered. Blocking or fluorescent quenching reactions comparing homologous and heterologous antisera have been partially successful and improvements in these techniques are being investigated. Greater sensitivity seems to be evident when formalin fixation is employed, but an increase in false positives is also noted. Ethanol fixation reduced the number of false positives but with a reduction in fluorescent intensity.

Although no claims are made for this procedure at this time, preliminary tests would indicate that a fluorescent spot test for the detection of staphylococcal enterotoxin (PB-9-B) is feasible, will have reasonable sensitivity and can be easily accomplished.

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ENDOTOXIN IN TYPHOID FEVER AND TULAREMIA IN MAN

Sheldon Greisman, M.D.*

Previously, we have reported that volunteers infected with Salmonella typhosa or Pasteurella tularensis develop a remarkable hyperreactivity to the pyrogenic activity of various endotoxin preparations. This hyperreactivity commences during the incubation period, one or more days prior to the onset of overt illness, and persists into the initial afebrile convalescent day. Thereafter, progressive decrease in reactivity develops until by the third afebrile day, reactivity decreases below baseline and tolerance is apparent. When volunteers are rendered tolerant to endotoxin prior to infection, this tolerance is suppressed and hyperreactivity is observed as in those volunteers not previously rendered tolerant. The mechanism for this remarkable increase in reactivity to endotoxin during typhoid fever and tularemia has been under continued study. We may summarize our present knowledge as follows:

The hyperreactivity is not related to nonspecific effects of infection or fever per se. The latter was evidenced by the exaggerated reactions occurring prior to the onset of overt illness. Additional control studies in volunteers rendered febrile by infection with the virus of sandfly fever indicated no suppression of preinduced tolerance to endotoxin and no hyperreactivity in those volunteers without preinduced tolerance.

The hyperreactivity is not attributable to impairment of generalized reticuloendothelial (RES) phagocytic activity. RES phagocytic activity was assessed with I^{131}-labeled aggregated human serum albumin with the collaboration of Dr. Henry N. Wagner, Jr., of the Johns Hopkins School of Medicine. No depression of the rate of clearance of this colloid from the blood was seen during the period of hyperreactivity to endotoxin. Indeed, enhanced clearance of the aggregated albumin was consistently observed during this time.

Obviously, studies of generalized RES phagocytic activity, as measured with aggregated albumin, may not reflect specific alterations in clearance of endotoxin per se by the RES. Studies with Cr^{51}-labeled Pseudomonas endotoxin in 8 volunteers are now in progress and have thus far demonstrated a significant acceleration of clearance rates in normal man as tolerance is established by daily intravenous injections. In one subject, there was one dissociation of clearance and the pyrogenic reaction, i.e., an increased febrile response occurred on day 2 associated

* Division of Infectious Diseases, University of Maryland School of Medicine, Baltimore, Maryland.

with an acceleration of clearance. With this one day exception, the acquisition of pyrogenic tolerance was associated with progressive acceleration of clearance of the Cr^{51}-labeled endotoxin. These findings contrast with the absence of accelerated clearance of I^{131}-labeled aggregated albumin during the acquisition of endotoxin tolerance in man. Such data support the concept that tolerance in man is specific and is based, at least in part, upon the production of antibody that facilitates the uptake of endotoxin by the RES. Studies are currently in progress to determine the clearance rates of isotope-labeled endotoxin during the phase of typhoid fever and tularemia when tolerance is suppressed and hyperreactivity to endotoxin ensues.

CURRENT STATUS OF CLINICAL TYPHOID FEVER STUDIES

Richard B. Hornick, M.D.*

Results of the mass field trials of typhoid vaccines conducted in Yugoslavia and British Guinea are not available. Each of these studies was well controlled, involved large population groups, and employed identical vaccines; epidemiological studies were thorough in each but seemingly divergent results emerged. In Yugoslavia, vaccine L, the heat-killed, phenol-preserved vaccine was slightly more effective than vaccine K, the alcoholized vaccine, in protecting against typhoid fever. Each vaccine was significantly superior to the control Shigella vaccine, demonstrating that typhoid vaccines may lower the incidence of the disease in an endemic area but not eliminate it. On the other hand, vaccine K, acetone-treated in this study, was definitely more efficacious in British Guinea than vaccine L. Both were superior to the control tetanus vaccine. Russian investigators have demonstrated the usefulness of alcoholized vaccine in school children compared to nonvaccinated controls. Other Soviet workers have shown that the use of a chemically adsorbed vaccine was associated with a lower attack rate of typhoid fever than that following alcoholized vaccine usage in a comparable group.

One may cautiously conclude from all these studies that vaccines probably lower the attack rate of typhoid fever but confusion still exists as to the individual value of each product in various age groups and geographic locations. This brief review of recent field trials with typhoid vaccine points up the value of volunteer studies. Through the evaluation of induced disease, it is hoped that specific assessments of challenge dosage, strain stability and quantitation of host defense mechanisms will lead to a clearer understanding of the role of typhoid vaccine in preventing or ameliorating typhoid fever.

Initial volunteer studies established that a reproducible disease could be produced with the Quailes strain of Salmonella typhosa when 10^9 organisms were given by mouth. Further challenge studies were necessary to establish the dose response for man so that an appropriate infecting inoculum could be applied to vaccine evaluation. Results of these studies are outlined in Table I. Mild, self-limiting disease, occasionally associated with bacteremia, is not included in the numerator. Accumulated percentages of infections for each dose are listed in descending order in the last column. These figures do not include volunteers vaccinated as part of our study, those made tolerant to endotoxin prior to infection, or cases of reinfection typhoid. The majority of volunteers have served in the

* Division of Infectious Diseases, University of Maryland School of Medicine, Baltimore, Maryland.

TABLE I. DOSE RESPONSE FOR MAN OF SALMONELLA TYPHOSA (Reed-Meunch Method)

DOSE	NO. TREATED/NO. EXPOSED	% INFECTED
10^9	31/33	98
10^8	8/9	87
10^7	10/14	70
10^5	4/12	21
10^3	0/12	0

Armed Forces and are presumed to have received typhoid vaccine previously. Incidentally, all subjects made tolerant to endotoxin by Dr. Greisman have developed typhoid fever following a challenge of 10^9-cells despite the presence of greatly elevated "O" agglutinin titers subsequent to the intravenous administration of homologous and heterologous endotoxins. The ID_{50} for the Quailes strain in man is approximately 10^6 organisms.

Initial challenge studies of small numbers of vaccinated volunteers suggested that a 1,000 ID_{50} dose or 10^9 cells was overwhelming. It was decided that a lower challenge dose would be employed to demonstrate immunity and if such could be shown, then return subsequently to the larger doses. A 10 ID_{50} dose was selected. Small numbers of men have been studied to date which precludes definite conclusions. Results of the small challenge studies are illustrated in Table II. The 10^7-cell challenge appears to be satisfactory

TABLE II. PROTECTION AFFORDED BY VARIOUS VACCINES TO TYPHOID VACCINE CHALLENGE

VACCINE	NO. TREATED/NO. EXPOSED	
	10^9 cells	10^7 cells
Acetone K	2/3	2/6
Phenol L	3/4	3/5
TAB	1/1	5/6
Vi	6/7	1/3
Control	7/7	10/14

because infection rate in the controls remains at 70% and benefits from vaccination should become apparent as more men are studied. The majority of the volunteers listed in the 10^9-cell challenge received their last of 3 inoculations of K, L or TAB vaccines 3 months prior to challenge. The Vi vaccinees were challenged at 1-4 months in most cases. In this small group of volunteers no appreciable protection could be demonstrated following a 10^9-cell challenge.

Although the number of volunteers challenged with 7 logs of organisms is similarly small, it is suggested that differentiation in effectiveness among the vaccines may be established. The interval between original vaccination and challenge was considerably longer for this group, i.e., > 14 months. A few of these subjects received booster doses of vaccine 6-8 months prior to challenge. Two of the 5 men who received K vaccine were given booster shots and one became ill. One of the 2 L vaccinees and 2 of 3 TAB vaccinees who were given additional doses of vaccine developed typhoid fever.

Significant elevations in agglutinin titers occurred in all those men who were treated for their infection. Only one volunteer who had received K vaccine and experienced a self-limiting disease was found to have an 8-fold increase in O agglutinins. Those vaccinees without any clinical evidence of disease failed to demonstrate changes in antibody titer following the ingestion of viable organisms.

Illness in the vaccinated volunteers was as severe as that observed in the controls, whether the infecting dose was 10^9 or 10^7 cells.

Previously, the typhoid fever infections of 2 volunteers following second exposures to S. typhosa were presented to Commission members. Prior experience with a 10^9-cell challenge in one did not prevent disease on rechallenge with the same dose 6 months later. Eight volunteers have recently ingested another dose of bacteria. Table III presents their prior exposure experience and subsequent clinical course. Previous challenges were 10^9, 10^5 and 10^3 cells.

TABLE III. RESULTS OF RECHALLENGE (10^7 S. typhosa) STUDY IN VOLUNTEERS

PREVIOUS EXPOSURE	NO. PREVIOUS DISEASE/TOTAL	NO. REINFECTED/TOTAL	NO. TREATED / NO. INFECTED
10^9	3/3	2/3	1/2
10^5	0/3	1/3	1/1
10^3	0/2	2/2	2/2

Initially, 3 men had received 10^9 organisms and experienced moderately severe typhoid fever. Each was treated with chloramphenicol. On rechallenge with 10^7 cells, 2 developed recurrent disease but only one severe enough to warrant therapy. Five inmates had participated in the studies to determine the ID_{50} and received either 10^5 or 10^3 S. typhosa. None of the 5 were clinically ill following these small oral challenges. However, some evidence of infection was apparent, in that one had had a positive blood culture and another a significant increase in "H" agglutinins. This group of 5 men was given the 10 ID_{50} dose of 10^7 cells and 3 became ill, requiring chloramphenicol to control the infection. The attack rate for the whole group is the expected response following such an oral challenge of controls. In this series there was no apparent protection following the first infection that could be demonstrated at time of rechallenge with a 10^6-cell dose.

Attempts to define the role of the tonsils as a portal of entry for S. typhosa have been initiated. Two volunteers were given a 10^9-dose of bacteria in milk to gargle. They expectorated the solution into sputum jars. In each instance over 90% of the inoculum was recovered. Both volunteers subsequently developed typhoid fever but the incubation period was prolonged. Chloramphenicol therapy was given. One subject subsequently relapsed. It could not be determined how much of the inoculum gained entrance via the tonsils or was swallowed. Four other volunteers were infected by placing the organisms in the stomach via a Levine tube. Each man experienced the usual degree of pharyngitis observed when illness is induced by gargling. Apparently, involvement of the tonsils and pharynx is not the direct result of local invasion but rather a manifestation of the generalized hyperreactivity of the reticuloendothelial system that occurs early in the disease. The latter has been measured by I^{131}-tagged heated human albumin technique developed by Drs. Wagner and Iio of Johns Hopkins Hospital.

Gastric survival of S. typhosa is largely unknown as a factor in the pathogenesis of typhoid fever. In the current studies, aliquots of gastric aspirate were removed just prior to challenge and at 15 min-intervals for 1 hr. This was cultured quantitatively and pH determinations performed. One of the 4 men showed positive cultures at 15 and 30 min. Four and 2 logs of organisms were recovered per milliliter of gastric juice at these time intervals. There is obvious irregular gastric distribution of the bacteria so that examining one small sample in the vicinity of the tube tip is grossly inadequate. It appears that the contaminated milk used as the inoculum is associated with viable bacilli in the stomach for at least 30 min. The amount of milk used did not appreciably alter the pH of the gastric samples.

Since chloramphenicol remains the sole antibiotic that is predictably effective for treatment of typhoid fever, it was important to evaluate other potentially effective antibiotics showing in vitro activity for S. typhosa. To date, Ampicillin, Humatin, Colymycin and Gentamycin have been appraised in small numbers of infected volunteers. In no instance were these antibiotics successful in controlling the infection and ameliorating the clinical manifestations during the period of observation. Each drug was given for

56 to 96 hr before it was deemed necessary, on clinical grounds, to discontinue the experimental agent and administer chloramphenicol. This substitution uniformly resulted in prompt clinical response. It would appear that these newer antimicrobial drugs are less effective than chloramphenicol in reducing the bacterial population in the host. Other antibiotics will be tried as promising ones become available. These clinical studies alone attest to the value of this volunteer program since additional appraisal of these antibiotics in patients are unnecessary. Hence, serious sequelae and possible deaths from typhoid fever are averted.

In the next month, concurrent with aerogenic tularemia studies, a few men will be exposed to small doses of aerosolized S. typhosa. This route of infection will be investigated further on the basis of preliminary findings.

During the past month 170 men have been vaccinated with K, L and Vi vaccines. Challenge of these men will probably begin in October and November. It is hoped that this experience will provide a definitive answer on the effectiveness of vaccines in induced typhoid fever.

EFFECT OF VIRUS INFECTION ON HOST CELL PROTEIN SYNTHESIS

Jerry R. Mohrig, 1st Lt., MSC*

An understanding of the mechanism of the pathogenic effect of a virus infection, fundamentally, may well come from a knowledge of its effect upon host cell metabolism. The capability of the host to synthesize protein in virus infection may be basic to the problem of the retention of cellular integrity. In the past few years, many workers have studied in vitro cell-free protein synthesis as measured by the incorporation of C^{14}-labeled amino acids into cellular protein fractions. From these studies, a great deal of information pertaining to the intermediate processes of cellular protein synthesis has been obtained. The importance of cellular ribonucleic acid (RNA) in protein synthesis is well known. Venezuelan equine encephalomyelitis (VEE) is an RNA virus which in the mouse manifests its virulence through central nervous system involvement. Therefore, the effect upon protein biosynthesis by microsomal particles obtained from brain cells of mice infected with VEE has been studied.

Two years ago, Colonel Irving Gray reported to this Commission the details of a system which allowed the uptake of C^{14}-labeled leucine into the microsomal fraction of mouse brains.[1] This procedure involves differential centrifugation of the homogenized cell extracts into microsome and enzyme fractions following the procedure of Keller and Zamecnik.[2] A series of experiments has been completed which shows the changes in protein synthesis encountered in these microsomal preparations obtained from brains of mice infected with Trinidad strain VEE, when incubated in vitro with the soluble pH 5 enzyme fraction from the same tissue, in addition to adenosine triphosphate, guanosine triphosphate, phosphoenol-pyruvate, pyruvate-kinase, magnesium chloride, and uniformly labeled L-leucine-C^{14}. The result of these experiments is the substantial stimulation of protein synthesis in mice infected with VEE virus when compared to uninfected mice. This stimulation is greatest on the second day after inoculation, whereas the central nervous system involvement of this viral disease does not become apparent until day 4 following inoculation. The increase in protein synthesis occurs during the time when the virus titer is increasing in the brain. However, the subsequent drop in synthesis ability, which is observed as central nervous system involvement becomes apparent, occurs while the virus titer is remaining at a high level.

By contrast, protein biosynthesis is not stimulated in mouse brain microsomal preparations obtained from mice infected with attenuated strain VEE virus. The results of 2 replicate experiments, in which the protein synthesis ability of microsomal preparations obtained each day for 6 days following inoculation was measured, show that all C^{14} incorporation values obtained

* U. S. Army Medical Unit, Fort Detrick, Maryland.

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STATUS OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VACCINE

Thomas J. Smith, Major, MC*

As described at last year's CES meeting, the incidence of reaction in humans to the attenuated Venezuelan equine encephalomyelitis (VEE) virus led to the development of a seed virus at the 81st tissue culture passage level. The inoculation of this material into various species of animals resulted in fewer adverse effects than did the older vaccine preparation. This was accomplished by passage of 78th passage VEE virus into a chick fibroblast culture, at which time plaques were selected for further passage on the basis of mouse virulence. The selected virus was then passaged twice in guinea pig heart cell culture, the growth media containing 0.5% human serum albumin. Previously bovine serum albumin had been employed. This plaque-derived virus grown in human serum albumin, while undergoing no apparent loss in antigenicity, caused fewer adverse effects in guinea pigs, monkeys, burros and man than did previous VEE vaccines. A lot of vaccine, TC-82 Lot 2-9, was prepared from the seed virus and used as an immunizing agent. The serologic responses in 998 individuals inoculated with this vaccine are summarized in Table I.

TABLE I. SEROLOGIC RESPONSES IN 998 INDIVIDUALS TO SUBCUTANEOUS INOCULATION OF ATTENUATED VEE VIRUS (TC-82 LOT 2-9)

NUMBER OF INDIVIDUALS	RECIPROCAL HI TITER Preimmunization	% DEMONSTRATING RISE IN TITER (4-fold or >)
409	< 10	93.4
125	10	80.8
162	20	50.0
120	40	25.0
92	80	5.5
47	160	4.3
23	320	0
12	640	0
3	1280	0
2	2560	0
2	5120	0
1	10240	0

The estimated dose was 1,000 guinea pig intraperitoneal infectious dose$_{50}$ (GPIPID$_{50}$). All but 17 of these individuals had received the killed VEE virus vaccine at some time in the past. At least one member

* U. S. Army Medical Unit, Fort Detrick, Maryland.

of the group, with a preimmunization titer of 1:5120, had experienced an infection with the Trinidad strain of VEE virus. Others with high hemagglutination-inhibition (HI) titers may have incurred a VEE infection at some time, but this was not documented.

The group contained 409 individuals with a preimmunization HI titer of < 1:10; 93.4% of these people demonstrated a 4-fold or greater rise in titer following immunization. Of 125 individuals with a preimmunization titer of 1:10, 80.8% responded serologically. Fifty percent of 162 individuals with a preimmunization titer of 1:20 showed a rise in titer, while 25% of 120 individuals with titers of 1:40 showed some change. Only 5% of those with titers of 1:80 or 1:160 had an increased titer following inoculation, while no individuals with a preimmunization titer of 1:320 or greater showed a change.

This group of vaccinees was not subjected to detailed clinical study following inoculation, so that minor symptoms related to the vaccine undoubtedly went unreported. Two people did present themselves for medical care because of fever and headache.

An additional 420 individuals were inoculated with the same virus preparation at Dugway Proving Ground, and while serologic testing has not been completed, better than 95% of those tested have showed an increased HI titer following vaccination. This group contained a greater percentage of individuals with no prior immunological experience with VEE than did the Fort Detrick group. A total of 5 reactions were recorded in the Dugway group, with 2 resulting in a brief period of time lost from work.

Approximately 100 people with no immunological experience with VEE have been vaccinated with TC 82-2-9 at Fort Detrick. The standard subcutaneous dose has been 1,000 $GPIPID_{50}$. The recorded reaction rate is approximately 5 to 8%, and all reactions have been short-lived. All but 5 of this group responded to a single inoculum of vaccine with an HI titer ranging from 1:80 to 1:2560. Of the 5 people who did not respond to the primary inoculum, 3 developed HI titers when the same dosage was repeated One required a 3rd, and one a 4th inoculation to elicit a serologic response.

There have been no known occurrences of VEE infection in individuals who have received the live attenuated vaccine. Two subjects with HI titers of 1:40 following vaccination have been challenged with an estimated 80 $MICLD_{50}$ of Trinidad strain VEE virus. Absence of clinical illness and an unchanged HI titer 1 month postchallenge indicated protection against the virulent virus.

HI titers and serum neutralizing indices (SNI) have been determined in 7 individuals 24 months or longer following vaccination with live VEE vaccine. Results are shown in Table II. As can be seen HI and SNI antibodies persist for 24 months or more.

TABLE II. HI AND NEUTRALIZING TITER OF 7 PERSONS IMMUNIZED WITH LIVE VEE VACCINE

SUBJECT	RECIPROCAL HI TITER			SERUM NEUTRALIZATION INDEX
	Preinoculation	at 21 days	at >24 mon	at >24 mon
P.B.	10	1280	320	$\geq 1.6 \times 10^4$
H.D.	-[a]	160	320	2.2×10^3
R.M.	-	1280	160	1.3×10^2
W.S.	-	320	40	2.9×10^2
D.C.	-	80	160	1.9×10^3
A.B.	-	160	40	1.0×10^2
M.W.	-	2560	160	5.6×10^2

a. - equals < 10.

Since viremia is known to occur in animals and man following inoculation with attenuated VEE virus, and since it is known that the virus is capable of undergoing reversion to a virulent form under certain circumstances, such as serial intracerebral (IC) inoculation in mice, studies are in progress to investigate the question of possible mosquito transmission of the attenuated virus. These studies are continuing in cooperation with the Entomology Division of the U. S. Army Biological Laboratories at Fort Detrick.

Two species of mosquitoes, Aedes triseriatis and Aedes aegypti have been employed in these studies because of the high efficiency of infection and transmission of the Trinidad strain of VEE virus by these strains. The virus used has been 93rd passage level VEE, produced by the National Drug Company. This vaccine strain, propagated in guinea pig heart cell culture, was derived from the plaqued virus already mentioned, and will be described in greater detail later. Initially virus was inoculated into guinea pigs; A. triseriatus were fed on the infected animals. The peak level of viremia in guinea pigs was found to be less than 100 $GPIPID_{50}$/ml regardless of dose inoculated. Because of the low level of viremia, none of the mosquitoes, including those tested immediately after feeding, were infected.

Attempts were then made to infect A. triseriatus by the "hanging drop" method utilizing the attenuated virus in an equal volume of 1 M sucrose solution containing normal guinea pig erythrocytes. This suspension had a virus titer of $10^{-6.8}$ $GPIPID_{50}$/ml (Table III). Immediately and on days 5, 10 and 15 after feeding, each of 5 mosquitoes was triturated and the

TABLE III. INFECTION AND TRANSMISSION OF ATTENUATED VEE VIRUS BY A. TRISERIATUS

DAYS AFTER FEEDING	NO. INFECTED/NO. TESTED	NO. TRANSMITTING/NO. TESTED
0	5/5	Not done
5	3/5	Not done
10	2/5	Not done
15	2/5	Not done
20	10/35	1/35
35	4/20	1/20

supernate inoculated intraperitoneally (IP) into guinea pigs. Twenty and 35 days after feeding 35 and 20 mosquitoes, respectively, were individually fed on guinea pigs and then tested for virus as were the earlier samples. Fourteen days after inoculation or exposure all animals were challenged IP with approximately 1,000 GPIPLD_{50} of Trinidad VEE virus. Thirty percent of the mosquitoes were shown to be infected, while only 1 of the 10 shown to be infected on day 20 and 1 of the 4 on day 35 transmitted the virus to guinea pigs.

A second study employed both A. triseriatus and A. aegypti. On day 20 approximately half of A. triseriatus and 18% of A. aegypti were infected, while 2 of 15, and 2 of 6 respectively, transmitted the virus to guinea pigs (Table IV). The observed infection and transmission rates are low compared

TABLE IV. INFECTION OF A. TRISERIATUS AND A. AEGYPTI WITH ATTENUATED VEE VIRUS AND TRANSMISSION TO GUINEA PIGS

DAYS AFTER FEEDING	NO. INFECTED/TOTAL		NO. TRANSMITTING/NO. INFECTED	
	A. triseriatus	A. aegypti	A. triseriatus	A. aegypti
0	5/5	5/5	0/5	0/5
5	2/5	0/5	0/2	
10	4/5	0/5	0/4	
15	2/5	0/5	0/2	
20	15/29	6/33	2/15	2/6

to those obtained in other studies by Corristan at Fort Detrick employing A. triseriatus and the Trinidad strain of VEE virus. Under conditions comparable to those employed in these studies, 100% of A. triseriatus ingesting approximately 1 x $10^{4.5}$ $GPIPLD_{50}$ of Trinidad strain VEE virus were infected, with 95% transmitting. None of the animals inoculated with mosquito materials, or on which mosquitoes had fed, showed signs of illness. Based on these limited observations there was no evidence of a change in virulence of the virus following passage through mosquitoes.

A study being conducted by Dr. Work at Communicable Disease Center will provide further information regarding mosquito transmission of the attenuated virus. Mosquitoes are being fed on volunteers inoculated with the virus. Infectiousness and transmissibility of the virus in the mosquito will be measured and correlated with levels of viremia in the volunteers on whom the mosquitoes feed. This study is in progress and no data are available.

Subsequent to assay in animals and administration of the plaque-derived virus to man, the virus was passaged 10 additional times in guinea pig heart cell culture, with human serum albumin in the growth media. This was carried out to evaluate the stability of the virus in terms of virulence and antigenicity. It was found that both of these properties were unchanged from the original seed virus at the 91st passage level when tested in guinea pigs. A pool of 92nd passage virus was prepared by National Drug Company to serve as seed virus for vaccine production, and all lots of vaccine produced by that company are 93rd passage level virus. In initial assay with TC-93, again antigenicity appeared unchanged. Accordingly, a titration of the vaccine was conducted in volunteers.

Forty-two volunteers who had no known immunological experience with VEE were utilized in a study to determine the human ID_{50} of attenuated VEE virus (93rd passage National Drug Company Lot No. 4), and to correlate incidence of reactions with administered dose. The men were divided into 7 dosage groups of 6 men each and inoculated subcutaneously with serial 10-fold dilutions of the virus. It had been anticipated that dosage of virus would range from 100,000 to 0.1 $GPIPID_{50}$. On the basis of simultaneous titration of the virus in guinea pigs however, dosage in volunteers ranged from 7,400 down to 0.007 $GPIPID_{50}$.

Subjects were observed for 12 days following inoculation in a double blind study and all physical signs and symptoms recorded. Attempts at virus isolation from blood and pharyngeal washings were made on 3 occasions in each man during the 12-day period. HI titers were determined on sera obtained prior to virus administration and on days 14, 28 and 56 post-inoculation.

Table V shows the HI titers of these individuals following inoculation with the vaccine. All 6 in the highest dosage group responded, although some did so only to a minimal degree. One man in the second group showed

TABLE V. HI RESPONSE[a] IN HUMANS VACCINATED WITH ATTENUATED VEE (ND-4)

ESTIMATED DOSE $GPIPID_{50}$	SUBJECT	PREIMMUNIZATION	RECIPROCAL HI TITER ON DAYS		
			14	28	56
7400.0	1	-[b]	10	10	-
	2	-	-	10	-
	3	-	320	320	80
	4	-	40	80	40
	5	-	20	40	40
	6	-	20	20	20
740.0	1	-	20	10	-
	2	-	-	-	-
	3	-	10	20	20
	4	-	40	80	80
	5	-	20	80	80
	6	-	80	160	80
74.0	1	-	-	-	-
	2	-	80	160	160
	3	-	40	80	80
	4	-	-	20	20
	5	-	-	-	-
	6	-	-	160	80
7.4	1	-	20	80	80
	2	-	-	-	-
	3	-	-	-	-
	4	-	-	40	20
	5	-	-	40	40
	6	-	-	-	-

a. 0.74, 0.07 and 0.007 $GPIPID_{50}$ doses of live virus resulted in no HI response in each of 6 individuals.

b. - equals < 10.

no change in titer, while 2 in the 3rd group demonstrated no rise in titer. Half of the 4th group showed a serologic response, while none of the remaining groups appeared to be immunized.

There was a total of 12 reactions to the vaccine administration. The most common manifestations of illness were headache, fever, myalgia, eye pain, fatigue and weakness. Conjunctivitis was the most common abnormal physical finding. A mild leukopenia with a relative lymphocytosis was noted commonly. All reactions were short-lived and there were no sequelae. Virus was isolated from throat washings of 6 individuals, while viremia was noted in only one. These 6 subjects were distributed throughout the 4 groups receiving the highest vaccine dose. Virus was detected earliest on day 6, and latest on day 12. The volunteer in whom viremia was detected had a severe reaction of a biphasic nature, requiring hospitalization on day 6 because of high fever, headache, myalgia, anorexia, nausea, vomiting, and abdominal pain. He recovered in several days. Viremia was present on days 6 and 7; throat washings contained virus on days 6-10. A lumbar puncture performed on day 6 revealed a cell-free spinal fluid with normal protein and sugar content. No virus was cultured from the spinal fluid.

Table VI summarizes the clinical data and reactions obtained from this study.

TABLE VI. LABORATORY FINDINGS AND REACTIONS OF 42 VOLUNTEERS TO VARYING DOSES OF LIVE VEE VACCINE (ND-4)

DOSE OF VACCINE ADMINISTERED[a]	SEROLOGIC RESPONSE	REACTIONS	VIRUS IN THROAT WASHING	VIREMIA
$GPIPID_{50}$	No.	No.	No.	No.
7400.0	6	4	1	0
740.0	5	3	2	0
74.0	4	3	2	1
7.4	3	2	1	0
0.74	0	0	0	0
0.07	0	0	0	0
0.007	0	0	0	0

a. Seven groups of 6 volunteers each.

On the basis of HI response in this group of volunteers the vaccine contained 3.46×10^4 human ID_{50}/0.5 ml. Simultaneous guinea pig titration indicated that the preparation contained 7.4×10^5 $GPIPID_{50}$/0.5 ml. Thus, a difference in titer of greater than one log was noted when the two hosts were compared.

Twenty-six National Drug Company personnel have been inoculated with 93rd passage attenuated VEE vaccine (the same preparation used in the previous titration). Table VII shows the HI responses. One of the group, T.O., had

TABLE VII. VEE HI ANTIBODY RESPONSE TO VEE VACCINE (93RD PASSAGE ND-4)

SUBJECT	RECIPROCAL HI TITER	
	Preimmunization	Postimmunization
T.O.	30	60
R.W.	640	1920
D.C.	60	60
S.R.	-[a]	3840
W.B.	-	3840
R.H.	-	960
W.T.	-	480
R.P.	-	240
K.G.	-	120
J.S.	-	120
P.K.	-	120
S.D.	-	60
J.D.	-	30
J.M.	-	30
K.M.	-	30
D.M.	-	30
R.C.	-	-
R.H.	-	-
G.D.	-	-
W.H.	-	-
S.C.	-	-
K.R.	-	-
F.L.	-	-
F.F.	-	-
C.T.	-	-
D.S.	-	-

a. - equals < 15.

received killed vaccine previously. Two others, R.W., and D.C., apparently had immunological experience with VEE from laboratory exposure. The remainder had no demonstrable HI antibody prior to inoculation. All received an estimated 1,000 tissue culture ID_{50}; the vaccine was not titrated in guinea pigs. Of the 23 individuals with no HI titer prior to vaccination, 10, or 44%, failed to respond to the inoculation with a rise in titer.

Likewise, a poor serologic response was noted in 6 monkeys receiving an estimated dose of $10^{6.6}$ tissue culture ID_{50} subcutaneously. Only 1 of 6 developed a HI titer.

The ability of the 93rd passage attenuated virus to infect animals via the respiratory tract was investigated at Fort Detrick, and again, a poor serologic response was noted.

Eight monkeys and 16 guinea pigs were exposed to aerosolized vaccine. The dosage in guinea pigs ranged from 4,800 to 20,000 $GPIPID_{50}$, while the range in monkeys was from 48,000 to 112,000 $GPIPID_{50}$'s. All guinea pigs were found to be protected against challenge with virulent VEE virus. Half of the monkeys however, had no demonstrable HI titer 21 days following aerosol exposure (Table VIII). Subsequent challenge with virulent VEE virus resulted in death in 1 of these 4 and a high HI titer in the remainder.

TABLE VIII. AEROGENIC IMMUNIZATION WITH 93RD PASSAGE ATTENUATED VEE VIRUS (ND-4)

INHALED DOSE $GPIPID_{50}$	NO. IMMUNIZED/NO. EXPOSED
Guinea pigs	
4,800 - 20,000	16/16
Monkeys	
48,000 - 112,000	4/8

In contrast to this, in an earlier study[1/] with aerosolized attenuated VEE virus in monkeys, all monkeys exposed to a dose of 3,000 $GPIPID_{50}$ or greater developed an immunizing infection. Viruses tested were both 50th and 80th tissue culture passage strains.

The apparent loss of antigenicity of the 93rd tissue culture passage VEE vaccine in man and monkeys has resulted in an investigation of possible causes by the National Drug Company.

This study is in progress and consists in producing 2 parallel passage lines of virus. Both will originate from the same seed virus, namely, VEE virus which has undergone 78 passages in guinea pig heart cells, one passage in chick fibroblast cells during which plaques were selected, and two further passages in guinea pig heart cell culture. This 81st passage level virus will be subjected to further passage in guinea pig heart cells, up to 93rd passage. Growth media will consist of Hanks' balanced salt solution and 0.5% bovine serum albumin for one passage line, and Hanks' balanced

salt solution with 0.5% human serum albumin for the other. The antigenic potency of both virus preparations at the 82nd and 93rd passage levels will be tested by animal inoculation followed by challenge with virulent VEE virus. If significant differences in potency are noted, the remaining passage levels will be tested.

In the interim, a sufficient quantity of attenutated virus has been produced and tested at Fort Detrick to provide for immunization of people at risk to VEE infection.

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BROAD SPECTRUM CHEMOPROPHYLAXIS OF TYPHOID FEVER AND TULAREMIA

Richard B. Hornick, M.D.*

Typhoid fever and tularemia are members of a group of diseases characterized by intracellular parasitism. Immune mechanisms responsible for eradication of these infections are largely unknown. Furthermore, the protection which the host provides Salmonella typhosa and Pasteurella tularensis in this intracellular environment probably accounts for recurrences under natural conditions and for even higher relapse rates associated with broad spectrum bacteriostatic antibiotic therapy. Both of these organisms grow well in tissue culture systems. Such an experimental approach can be adapted to resolving problems associated with therapy of these infections. Smadel's group[1,2] showed that S. typhosa propagate well in mouse fibroblasts and observed the effect of various antibiotics on intra- and extracellular survival of these organisms. These studies demonstrated that these bacteria ceased to multiply when streptomycin, chloramphenicol, penicillin or Synematin B were added to infected tissue cultures. Cessation of extra- and intracellular multiplication occurred simultaneously suggesting successful intracellular penetration of antibiotic. In this tissue culture system, exposure to chloramphenicol for 21-28 days was necessary for bacterial sterility. However, in some instances late relapses occurred despite negative culture in the interval following elimination of the antibiotic. It was postulated that these organisms were in a latent phase within the cell or that a few bacteria were missed on earlier culture attempts. The resemblance of these studies to the relapse and human carrier state is worth emphasis. These tissue culture studies showed striking reduction of bacterial population in the log phase of growth; thus it appears that early institution of antibiotic therapy following infection should eradicate or eliminate most invading organisms. If the dose of antibiotic could be so regulated as to prevent overt disease and yet allow subclinical infection to occur, some concept of immune relationship might be ascertained.

It seemed important, therefore, to conduct chemoprophylactic studies in volunteers infected with P. tularensis and S. typhosa. Our experience with such studies will be presented here. Major Sawyer and his group have completed and are contemplating detailed studies of tularemia in volunteers. These results are awaited eagerly.

McCrumb[3] demonstrated that streptomycin was effective in preventing ulceroglandular tularemia in 9 of 10 volunteers. The antibiotic was given for 5 days starting 1 hr postinfection. Chloramphenicol therapy for a

* Division of Infectious Diseases, University of Maryland School of Medicine, Baltimore, Maryland.

similar period was less effective, since 5 of 7 volunteers manifested disease after a delayed incubation period. Only those subjects who manifested overt disease developed agglutinins. Therefore, when prophylactic antibiotic prevented illness, it did so by completely inhibiting antibody formation.

More recently, as part of a larger study evaluating the immunoprophylaxis of living tularemia vaccine (LVS), volunteers have been infected with SCHU-S4 strain of P. tularensis and treated prophylactically with streptomycin. The earlier work of McCrumb revealed that 5 days of streptomycin (total dose of 10 gm) was very effective in preventing disease. Therefore, smaller dose regimens were appraised. Ten male volunteers were given 2,500 SCHU-S4 cells intradermally and 1 hr later 0.5 gm of streptomin intramuscularly. Concurrently, 2 other groups of 10 men each were infected. One group was composed of LVS vaccinated volunteers and the remaining 10 men served as nonimmunized controls. These 3 groups developed an interesting spectrum of clinical infections. For instance, within 24-48 hours the controls had small erythematous, papular lesions and were febrile. Simultaneously, the vaccinated men showed markedly positive skin tests at the site of inoculation. This lesion differed markedly from the controls; there was only one with induration and erythema which did not ulcerate as the controls. Those men who received streptomycin prophylactically had no evidence of disease during this period. It is emphasized that only one single 0.5-gm dose of streptomycin was administered 1 hr after infection. No evidence of disease appeared in any of these volunteers for 7 days. At that time small papular lesions developed and fever began. Ultimately, 9 of these men were treated with streptomycin for delayed infection. As a whole this group had less constitutional symptoms than the controls. Observed in Figure 1 that serological responses correlated with the clinical state, a 1-week delay in incubation period and in development of antibodies when contrasted with the controls. There were 2 volunteers in the chemoprophylactic group who showed no elevation of antibody titer. One was treated for a relapse because of systemic complaints and a minor local skin lesion in spite of normal temperature. The other untreated individual did not relapse. All 10 controls were infected and treated.

Table I presents the results of the trial just described and an additional study. The second study differed in the amount of inoculum, dose of streptomycin and interval of administering antibiotic after challenge. The results of the 2 series are similar including the clinical course. The incubation period was delayed for 1 week by the single 2.0-gm dose of streptomycin given 24 hr postinfection. Nine of the 10 men were treated eventually and all developed tularemia agglutinins. These studies indicate that a few bacilli survive the initial effects of early streptomycin therapy and subsequently cause overt disease. The factors concerned with the prolongation of the incubation period for 7 days are unknown.

Future trials will provide larger doses of streptomycin given shortly after infection. A single 0.5-gm dose is insufficient to completely suppress the infection after 1 hr when a moderate infecting dose is given.

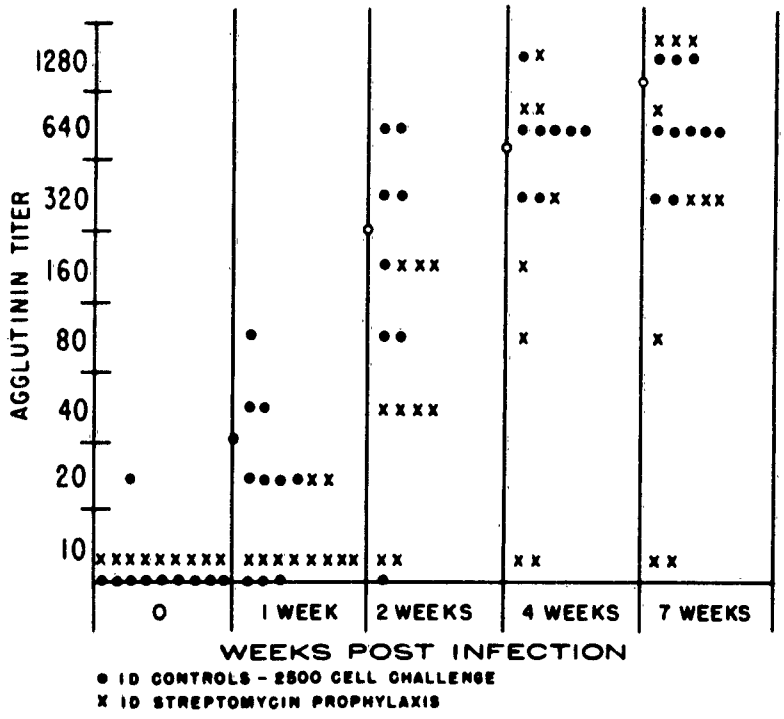


FIGURE 1. TULAREMIA ANTIBODY STATUS OF MEN GIVEN ONE 0.5-gm. DOSE OF STREPTOMYCIN 1 HR POSTINFECTION.

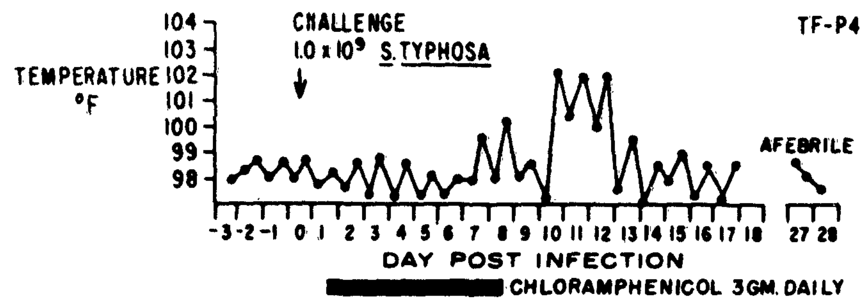
TABLE I. TULAREMIA CHEMOPROPHYLAXIS STUDY

INTRA-DERMAL DOSE	INTERVAL	SINGLE DOSE OF ANTIBIOTIC gm	NO. WITH RELAPSE TOTAL	NO. WITH SEROCONVERSION TOTAL
2,500	60 min	0.5	9/10	8/10
10,000	24 hr	2.0	9/10	10/10
TOTALS			18/20	18/20

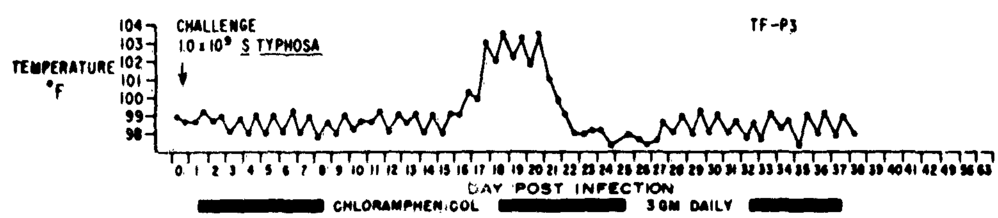
Studies of chemoprophylaxis in human typhoid fever are limited. Four volunteers were studied. Twenty-four hours after ingestion of 10^9 cells, each received 3.0 gm of chloramphenicol therapy daily in divided doses, 2 for 7 days and 2 for 28 days. Two control subjects and 1 treated for 7 days became clinically ill whereas 28 days of treatment suppressed infection. These findings coincide with the results of tissue culture work described above. Some interesting features evolved from this study. Figure 2 depicts the course of one volunteer who received 7 days of therapy. Although he was ill and febrile at the end of his chemoprophylaxis period, bacteriologic and serologic studies were negative for evidence of typhoid fever. He was not treated and remained well. His partner's clinical course is shown in Figure 3. Of particular interest was the presence of bacteremia during the latter stages of chloramphenicol therapy when his typhoid O and H antibodies also rose strikingly. The majority of volunteers infected with 10^9 organisms become ill on day 6 with concurrent bacteremia and serological response; this is the average incubation period. Bacteremia occurs simultaneously with the onset of fever. No further increase in antibody titers occurred after the onset of disease. The second volunteer demonstrated that 7 days of chloramphenicol therapy failed to eradicate all invading organisms. The antibiotic did not prevent bacteremia or antibody snythesis in the week postinfection.

The final two figures outline the clinical course of the two volunteers who received chloramphenicol continuously for 4 weeks. Figure 4 depicts the course of the first in whom no serologic evidence of typhoid infection was found. Stools were positive during the first 5 days indicating multiplication in the gastrointestinal tract. Serial studies showed no increase in antibody titer. The fourth patient (Figure 5) remained well although there was a significant rise in O agglutinins. Cultures were negative except for the initial positive stool culture.

Two control patients infected simultaneously developed typhoid fever and were treated with chloramphenicol. This experence with these few patients suggests that prolonged antibiotic chemoprophylaxis can prevent the development of typhoid fever. During the first week of therapy viable organisms

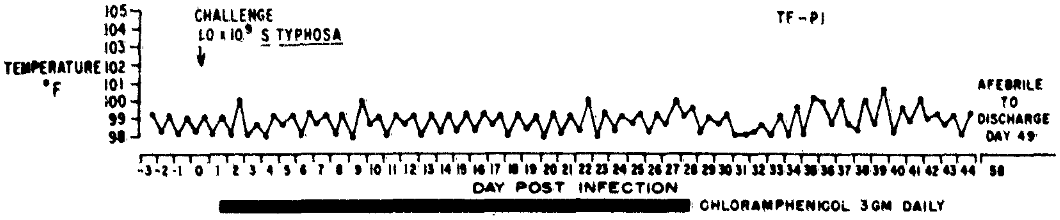


CULTURES	BLOOD	0 0 0 0 0 0 0 0 0 0 0 0
	STOOL	+ 0 0 0 0
ANTIBODY TITERS	O	40 80 80
	H	160 80 160
WBC		6950 9700 9600 4000
HCT		49 47 48
WEIGHT POUNDS		176 176 170 169

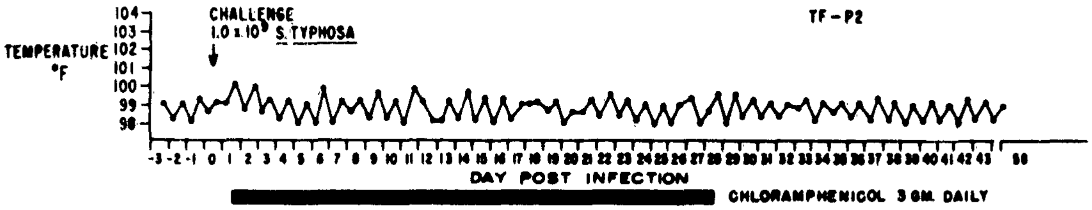


CULTURES	BLOOD	0 + + 0 0 0 0 0 0 0 + 0 0
	STOOL	+ 0 0 0 0 0 0
ANTIBODY TITERS	O	0 160 80 80 40 40 20 40 20 20
	H	0 320 5120 2560 1280 1280 640 160 640 640
WBC		9300 10,500 10,750 9250 7800 6600 9800 8750 9100
HCT		51 50 48 49 52 52 51
WEIGHT POUNDS		168 168 169 169 171

FIGURES 2 (TF-P4) AND 3 (TF-P3) CLINICAL AND LABORATORY SUMMARIES OF TWO VOLUNTEERS GIVEN CHLORAMPHENICOL PROPHYLACTICALLY 3-gm. DAILY FOR 7 DAYS



CULTURES	BLOOD	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
	STOOL	+ 0 0 + 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
ANTIBODY TITERS	O	80 160 160 160 160 160 160 160
	H	160 160 160 160 160 160 160 160
WBC		9400 14,500 10,750 9100 7000 11,700 7250 10,600 9550
HCT		52.5 46.5 49 49 48 49 48 48 50
WEIGHT POUNDS		169 171 174 174 178 180 176 174 175 176



CULTURES	BLOOD	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
	STOOL	+ 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
ANTIBODY TITERS	O	80 640 1280 1280 1280 640 640 320
	H	320 320 320 320 320 320 640 320
WBC		9100 9600 10,160 8950 9600 6900 10,000 10,300 11,000 9800
HCT		56 55 55 53 55 56 53 53 54 53
WEIGHT POUNDS		167 168 168 170 171

FIGURES 4 (TF-P1) AND 5 (TF-P2) CLINICAL AND LABORATORY SUMMARIES OF TWO VOLUNTEERS GIVEN CHLORAMPHENICOL PROPHYLACTICALLY 3-gm DAILY FOR 28 DAYS

were isolated from the blood and antibody formation occurred. These findings coincide with the tissue culture studies mentioned above. Ability of typhoid bacilli to remain dormant or develop "antimicrobial indifference" may account for the resistance to therapy and yet promote antibody genesis.[4] Only prolonged exposure of infected tissue culture cells to antibiotics sterilized this system. Similarly, 28 days of antibiotic was sufficient to prevent disease in man.

Rechallenge of such patients would determine whether such controlled infection leads to immunity. Tissue culture studies by Ambrose and Coons[5] showed that chloramphenicol reduced antibody formation. Such findings could explain why continuous chemoprophylaxis may inhibit immunity. However, agglutinins developed in usual titer in this small group. It is well known that these antibodies bear little relationship to ability to resist infection.

Tissue culture studies and additional chemoprophylactic trials are contemplated to clarify the relationship of antigenic stimulation through suppressive therapy and active immunization in typhoid fever.

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BLOOD-FREE CULTURE MEDIUM FOR PASTEURELLA TULARENSIS

Hugh B. Tresselt, Ph.D.*

Earlier work in our laboratory resulted in (1) the demonstration that whole blood treated with Duolite provided an excellent medium for isolation and growth of Pasteurella tularensis strain SCHU-S4,[1] and (2) the development of Gaspar medium, a blood-containing medium which consistently produced colonies 1 mm in diameter after 30 hr incubation in a water saturated atmosphere.[2]

In our early experiments on the enhanced growth of P. tularensis, blood was an essential constituent of all media. Therefore, efforts to study further the mechanism of enhanced growth were centered on the contribution of blood to rapid growth of the organism. Extracts of blood were prepared and analyzed for their constituents and growth-promoting properties. Through these experiments, it was found that ferrous sulfate could substitute for blood in modified Gaspar medium. Exploitation of this idea led to the development of a variety of blood-free media which promoted rapid colonial development of the organism.

The simplest medium consisted of tryptose broth with thiamine, ferrous sulfate, reduced cystine and glucose. These constituents were found essential to all experimental media which provided for rapid growth of the organism. Better growth was obtained when histidine was added to this combination but, the storage stability was poor. The addition of potassium chloride, histidine and Tris buffer to the essential ingredients, provided for storage stability, and colonial growth as good as, or slightly better than Gaspar medium. Colonies were consistently 1 mm in diameter after 30 hr incubation at 37 C.

The incorporation of antibiotics into this new, transparent medium provided a selective growth medium for this organism from clinical specimens.

This new medium (called "T" medium) prepared as a broth, supported abundant growth from an inoculum as low as 1 organism/10 ml of medium. "T" medium was also adequate for the growth of other organisms of interest to our Division, including virulent and avirulent strains of Bacillus anthracis and Pasteurella pestis; it also supported growth of Brucella spp, Salmonella spp, Shigella spp, and Diplococcus pneumoniae.

The mechanism involved in the rapid growth of P. tularensis on artificial culture media is not as yet fully understood. Our studies have brought out several factors which appear to be of importance. These may be summarized as follows.

* U. S. Army Medical Unit, Fort Detrick, Maryland.

1. Early colonial development was the result of decreasing both the lag phase and generation time of the organism. This was accomplished, we think, by adjusting the uninoculated medium to effect changes which are ordinarily brought about by the metabolic activity of the organism. According to Snyder, et al[3] and Ransmeier and Stekol[4] one major change in media which support growth of P. tularensis was the reduction of cystine. This observation was confirmed by our studies. When an adequate amount of reduced cystine was supplied in the preparation of medium, growth was accelerated. The requirement for the reduced form of cystine for enhanced growth was readily demonstrated by the failure of the organism to respond when cystine was substituted for reduced cystine in "T" medium.

2. The reduction of cystine by the organism, or the addition of cysteine HCl to the medium brought about changes in the oxidation-reduction (O-R) potential. The results of experiments which were designed to study the effect of O-R potential on the growth response of P. tularensis indicated that:

a. In freshly prepared, uninoculated "T" or Gaspar media, the O-R potential was very low due to the addition of reduced cystine. The organism did not survive in this highly reduced medium. It was necessary to oxidize a considerable amount of cysteine HCl by permitting the medium to stand overnight before inoculating with the organism.

b. When blood-free enhancing medium was prepared as a broth, it was not necessary to hold the medium overnight before inoculation with the organism. The measurement of the O-R potential of broth medium indicated that cysteine HCl was quickly oxidized to a level compatible with growth.

c. If, however, cystine was substituted for cysteine HCl, and the O-R potential values were artificially made to mimic the same levels as when cysteine HCl was added to the medium, rapid growth did not take place.

The O-R per se, therefore, did not appear to influence the growth response of the organism. Enhanced growth appeared rather, to be related to the amount of cysteine initially addeded to the medium.

3. Cysteine HCl appeared to be specific in its growth promoting properties. Glutathione, thioglycollate, homocysteine, ergothioneine, and a variety of other sulfhydryl-containing compounds could not be substituted for it.

4. Cysteine HCl was not the only essential component in the enhancing media. Thiamine was also indispensable. Thiamine was supplied in growth enhancing media by the addition of red blood cells, by tryptose broth containing thiamine, or by the addition of thiamine HCl.

5. In blood-free medium, ferrous iron was also essential. Ferric iron could not be used as a substitute. The role of ferrous iron is not understood.

The peculiar, specific requirements for the production of medium which enhanced growth of this organism suggested the possibility of a reaction between cysteine HCl and some other ingredient in the medium. The ability of the organism to grow on enhancing medium over a broad range of moderate O-R levels, accentuated the peculiarity of the requirement for an extremely low initial O-R potential, brought about by the high concentration of cysteine HCl required to effect enhanced growth. The need for a storage stabilizer such as histidine and/or Tris buffer further supported the idea of a reaction between ingredients in the medium. It was postulated that thiamine and cysteine HCl formed thiamine cysteine disulfide, as described by Matsukawa and Yurugi,[5] and that this complex was a growth factor for P. tularensis, just as thiamine was shown to combine with pantetheine to form thiamine pantetheine disulfide, the growth factor for Lactobacillus fermenti.[6] The formation of this postulated complex may also further explain the need for the overnight incubation of solid medium.

Although this complex has been identified chromatographically in growth enhancing medium, it has not been isolated in the pure state. The assay of this complex and its effect on growth of P. tularensis has been hampered by the inability to remove selectively thiamine and cystine from tryptose broth. No substitute has been found for tryptose broth. The importance of a cysteine thiamine complex awaits the solution of these isolation and assay problems. Other experiments to determine the importance of cysteine and thiamine have revealed that the reaction was not a simple reduction of thiamine disulfide, because thioglycollate, as well as cysteine, reduced thiamine disulfide, yet thioglycollate could not substitute for cysteine.

Interest in this area continues, because early data indicated that the growth requirements for the fully virulent SCHU-S4 strain of P. tularensis are quite different from those of the nonvirulent vaccine strain. It now appears that these studies will provide useful information concerning the chemical factors in virulence of this organism.

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ELECTRONMICROSCOPY AS A DIAGNOSTIC TOOL

Anne Buzzell, Ph.D.

This presentation concerns what we hope will be a practical use of an assay procedure we have worked out for counting particles at low concentration in virus suspensions in the electron microscope. The method will be described first. At the end of the talk the problems that must be solved before the assay can be used as a practical diagnostic tool will be discussed. Howard Hegstad worked out many of the details of the assay method.

The assay is based on the procedure of Sharp[1/] in which the virus is centrifuged onto a block of agar; the virus is then stripped from the agar with a collodion film, which is then placed on an electron microscope grid. The film was formed by letting a drop of collodion run over the agar; this then was floated from the agar, virus-side down, onto distilled water. This procedure has a number of disadvantages: viruses may be adversely affected by exposure to an organic solvent or distilled water and small particles may be washed off the agar; the procedure is tedious and not highly reliable since the small pieces of film are barely visible on the water and are often lost.

The modified procedure avoids all of the difficulties just mentioned. A "formvar" film is made on a separate block of agar. Reliable high quality films, thin and free of holes, are made by letting several drops of 1.5% "formvar" solution in ethylene dichloride run rapidly down the block, tilted at an angle of 45°, to a piece of bibulous paper which immediately removes the excess fluid. The block is then placed film-side down on a rectangle of "Saran Wrap" tautly stretched over a frame made of 3 glass microscope slides taped together, the middle third of the central slide being cut out, as shown in Figure 1. The saran has a circular hole, centrally placed, slightly larger than an electron microscope grid. The frame is turned over and the agar block with the virus particles is placed over the hole in the saran. With an agar block on each side, the formvar film makes uniform contact with the agar surface and picks up the virus particles. Both agar blocks are then removed by inserting a scalpel under an edge and peeling the agar back from the saran. The formvar film is then transferred to an electron microscope grid as follows: on a large piece of glass, the small square of glass cut from the central microscope slide of the frame is mounted on masking tape with a central reference hole cut in the tape. A stack of 2 or 3 electron microscope grids is placed over the reference hole and held in place by a piece of saran smoothed down over the glass. The grid to be coated with formvar is placed on the mound. The frame is then fitted down over the square of glass and the formvar is stretched over the grid and adheres to it when the film is pricked around the edge of the grid to free it from the saran.

* U. S. Army Medical Unit, Fort Detrick, Maryland.

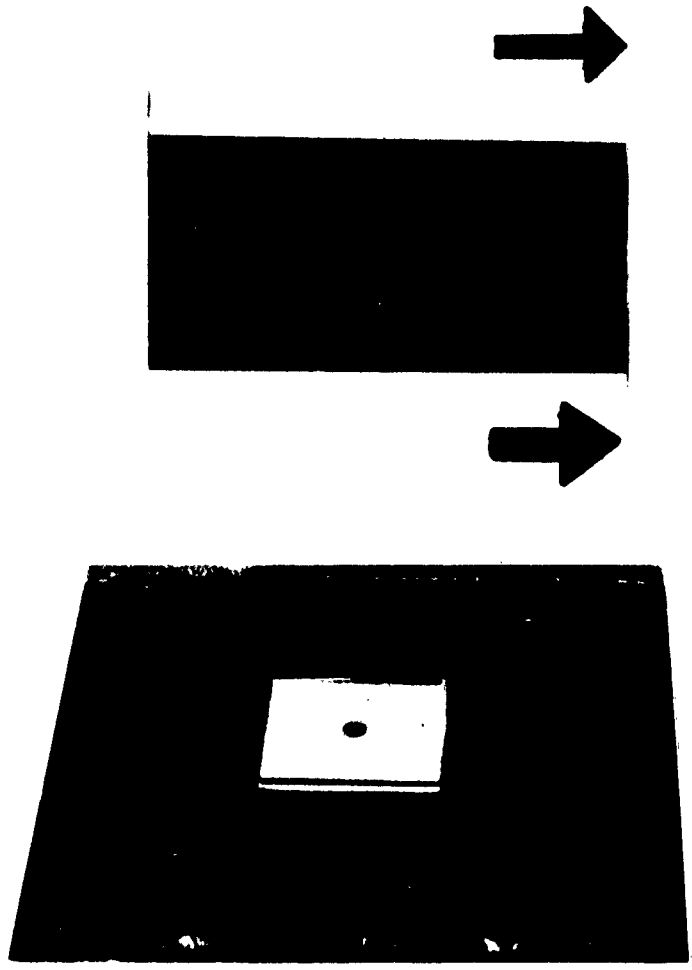

FIGURE 1. DEVICE FOR STRIPPING VIRUS PARTICLES FROM AGAR.

Recently Major T. J. Smith (U. S. Army Medical Unit) discovered that virus particles deposited on Millipore filters could be removed by pressing on the filter a piece of agar to which the virus sticks preferentially. This discovery increased considerably the sensitivity of the assay. The centrifugation method is limited by the capacity and dimensions of available centrifuge cells. For instance, with the cell we use, the capacity is about 0.6 ml and the agar area 0.6 cm^2. A 10^7 particle/ml of suspension containing virus will yield 10^7 particles/cm^2 on the agar. At 8,000 diameters magnification, a standard micrograph corresponds to a 4.0×10^{-7} cm^2 area of agar. At this magnification, the 10^7 particle/ml suspension would yield 4 particles per micrograph. By contrast, in the commercially available high pressure filtering device we use, fluid passes through the 1 cm^2 filter area; 10 ml of a 10^6 particle/ml suspension, or 100 ml of 10^5, can be used to give 4 particles per micrograph. At the maximum allowed pressure of 15 atmospheres, 10 ml will pass through the 10-20 mμ pore size filter required for virus retention in 20 min. The volume of fluid required could be cut at least 4-fold by designing a filtering device which used 0.25 cm^2 filter area. Since an electron microscope grid area is only 0.07 cm^2, 3 samples could still be obtained per filtration. A filtration requiring several hours, if done in the cold, should be no real hindrance to using solutions of 10^5 particles/ml. It should be pointed out also that infectious units generally comprise several hundred particles, so 10^5 particles/ml would usually correspond to 10^3 infectious units/ml or less.

To adapt this method for diagnosis, two principal problems must be solved. One is that serum does not readily pass through small pore size filters. It may prove necessary to separate the virus from the serum, for instance by ion exchange techniques. Urine, however, can be filtered as readily as water. The second problem is the obvious one of identifying the particles. The number of possibilities can be considerably narrowed down on the basis of morphology. Further narrowing might be achieved by negative staining which reveals certain internal and surface structures of virus particles. This approach has been proposed and explored to some extent by Smith and Melnick[2] and others. Whether the added information gained would warrant the extra trouble, for the negative staining technique has its difficulties, is a moot point at present. Final identification of the particles will probably require use of ferritin-labeled specific antibodies.

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RIFT VALLEY FEVER VACCINE

Raymond Randall, DVM*

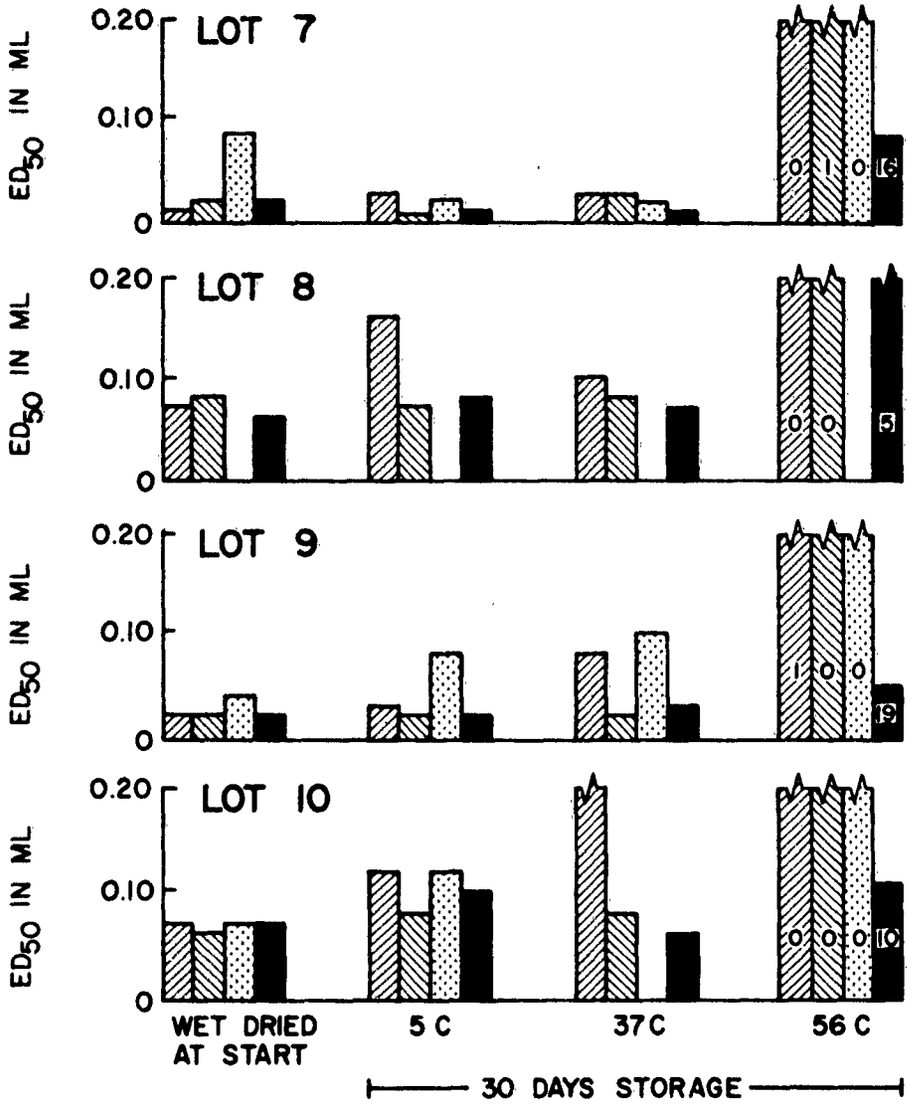
This report summarizes our studies on the evaluation of a stable lyophilized Rift Valley fever (RVF) virus vaccine. In addition the report covers the progress made to date for the commercial manufacture of RVF vaccine.

The need to protect persons at risk against RVF virus has led to the development and production of a safe and highly immunogenic formalin-inactivated vaccine. The vaccine currently being produced for use in man is prepared in African green monkey kidney cell cultures, maintained in Mixture 199, and infected with the field or pantropic strain of the virus. To date, more than 7,000 ml of vaccine have been distributed for use in more than 1,200 human beings. Included in this total are approximately 1,600 ml of the lyophilized vaccine. There have been no reports of any untoward reactions to either the fluid or lyophilized product.

Figure 1 summarizes studies on the storage stability of 4 lots of RVF fluid and lyophilized vaccines. In collaboration with personnel of the Department of Biologics Research, human serum albumin (HSA) was one of the additives tested as a stabilizer during lyophilization. This additive is not allergenic for man and has not been associated with viral hepatitis. The potency of fluid and lyophilized vaccines with and without the addition of 2% HSA was measured in mice by the antigen extinction test. Groups of animals were immunized with 0.2-ml amounts of serial 5-fold vaccine dilutions on days 0 and 7. The mice were challenged 7 days after the 2nd dose of vaccine with approximately 10,000 mouse LD_{50} of pantropic RVF virus. The potency of the vaccine is expressed as the effective dose 50% (ED_{50}), in ml/dose, required to protect 50% of the mice. The higher the column in the figure, the greater the amount of vaccine that was required to protect the mice and consequently the less potent the vaccine.

The first columns on the left show that the addition of 2% HSA did not alter the potency of RVF vaccine before or after freeze-drying since the ED_{50} values are essentially the same. After 1 month of storage at 5, 37, 56 C the potency of the vaccines was determined again. At 5 and 37 C, the middle columns on the figure, there was little change in the ED_{50} values. However at 56 C, the dried product containing HSA was significantly more stable than the liquid or the dried vaccines without HSA. This is clearly shown by observing the number of mice immunized with the undiluted vaccine that survived challenge. These numbers are presented within the bar graphs on the right. For example, with Lot No. 7 stored at 56 C, none or only 1 of 20 mice vaccinated with either liquid or dried vaccine without HSA

* Walter Reed Army Institute of Research, Washington, D. C.



Wet, no additive

Wet, 2 % Human serum albumen

Freeze dried, no additive

Freeze dried, 2 % Human serum albumen

Number of mice surviving 30 days after challenge with 10,000 LD_{50} of pantropic virus 7 days after second dose of undiluted vaccine— 20 mice in each group.

FIGURE I. STORAGE STABILITY OF FORMALINIZED RIFT VALLEY FEVER VACCINE.

survived challenge, whereas 16 of 20 vaccinated with the dried product containing HSA survived challenge. These data clearly show that the addition of HSA increases the heat and storage stability of the dried vaccine.

During a 15 month period, 7 replicate potency tests were made with Lot No. 12 lyophilized vaccine containing HSA to determine its suitability for use as a reference vaccine (Table I). The previously described antigen

TABLE I. REPLICATE ANTIGEN EXTINCTION TESTS OF LYOPHILIZED RIFT VALLEY FEVER VIRUS VACCINE IN MICE (LOT NO. 12)[a]

DATE ASSAYED	EFFECTIVE DOSE 50% (ED_{50} ml/dose)		LOG NEUTRALIZATION INDEX[b] 7 days postimmunization	
	Titration A	Titration B	Dose 1	Dose 2
14 March 62	0.013	0.023	2.0	2.5
4 April 62	0.009	0.009	1.6	3.6
17 April 62	0.013	0.008	Not done	Not done
26 June 63	0.020	0.018	1.7	2.9
20 Sept 62	0.007	0.009	1.3	3.8
22 May 63	0.005	0.003	2.4	2.9
11 June 63	0.006	0.008	2.4	2.8
	Geometric mean:	0.0095		

a. Lyophilized with 2% human serum albumin, USP.

b. Mice were vaccinated with undiluted vaccine on days 0 and 7.

extinction test was used in these assays. The individual ED_{50} values varied nearly 8-fold from 0.003 to 0.023 ml; however the average of each of the 7 assays varied less than 5-fold, i.e., from 0.004 to 0.019 ml. The maximum deviation from the geometric mean was less than 2.5-fold. These results indicate that a reproducible measure of the immunogenic capacity of the different lots of vaccine can be obtained by means of the antigen extinction test.

A comparison between the antigenic potency of fluid and lyophilized RVF vaccine were made in human beings receiving a primary 3-dose series or after a single booster dose in previously immunized persons (Table II). Lots 10

TABLE II. COMPARISON OF THE ANTIGENIC POTENCY OF FLUID AND LYOPHILIZED[a] RIFT VALLEY FEVER VIRUS VACCINE FOR MAN

LOT NO.	STATE	FOLLOWING PRIMARY 3 DOSE SERIES			FOLLOWING BOOSTER DOSE		
		No. with LNI[b] of 1.7 or >/total vaccinated		Mean LNI	No. with rise of LNI ≥1.0/ total boostered		Mean rise LNI
10	Fluid	17/25	(68%)	1.81	9/26	(35%)	0.62
	Lyophilized	11/18	(61%)	1.92	13/28	(47%)	0.91
11	Fluid	12/13	(92%)	2.9	11/15	(73%)	1.27
	Lyophilized	13/15	(87%)	2.4	12/15	(80%)	1.22

a. Contains 2% human serum albumin, USP.

b. Log neutralization index, the difference in the LD_{50} before and after immunization.

and 11 were employed in these experiments. Of the persons receiving the primary series of fluid vaccine Lot No. 11, 12 of the 13 persons or 92% developed a log neutralization index (LNI) of 1.7, or greater, while with the corresponding lyophilized vaccine containing HSA 13 of 15 persons or 87% responded equally well. The mean LNI of the persons receiving the fluid vaccine was 2.9 and for the lyophilized vaccine group 2.4. Of the persons receiving the booster dose, 11 of 15, or 73% for the fluid group and 12 of the 15, or 80% for the lyophilized vaccine group had a LNI rise of 1.0, or more. The mean increase of the LNI of the fluid group was 1.27 and for the dried vaccine group 1.22. Similar data are presented in the table for Lot No. 10 which was less potent.

Statistical analyses of these data show that there was no significant difference between the potency of the liquid or dried products when used either as a primary series or as a booster dose. It is of interest to note that among the persons boostered with Lot No. 10, 27 had received a primary series of Lot No. 4 fluid vaccine 2 years previously. None of these persons had any contact with the virus, yet each of these persons had a residual LNI of 1.7, or greater, prior to their current booster.

Further studies have been made of the primary antibody response in man to different lots of RVF vaccine. Vaccinees were bled prior to vaccination and 2 weeks after completion of a 3-dose series.

The data presented in Table III show the results obtained in man with each lot of vaccine. The results indicate that the several lots of vaccine

TABLE III. A COMPARISON OF THE RESPONSE OF MICE AND HUMAN BEINGS TO SEVERAL LOTS OF RIFT VALLEY FEVER VACCINE

VACCINE TESTED		MOUSE ANTIGEN EXTINCTION TEST (ED_{50} ml/dose)		PRIMARY HUMAN ANTIBODY RESPONSE[a]		MEAN LNI
Lot No.	State	Titration A	Titration B	No. with LNI[b] ≥1.7/ total vaccinated		
7	Fluid	0.025	0.028	15/16	(94%)	2.88
10	Fluid	0.115	0.093	17/27	(63%)	1.76
	Dry[c]	0.105	0.060	13/20	(65%)	2.02
11	Fluid	0.109	0.112	12/13	(92%)	2.90
	Dry[c]	0.043	0.053	34/49	(70%)	2.02
13	Fluid	0.010	0.012	38/41	(93%)	2.95
REFERENCE VACCINE						
12	Dry[c]	0.016	0.018			

a. Following a 3-dose series on days 0, 7, and 28.

b. Log neutralization index.

c. Human serum albumin.

could be divided into 2 groups: lots of moderate potency which induced effective neutralizing antibody in approximately 65% of the vaccinees, and lots of higher potency which converted more than 90% to a positive antibody status of log 1.7 or greater. The development of a serum LNI of 1.7 was arbitrarily established as the criterion of effective antibody conversion. The mean antibody level of the former vaccine lots was only 1/10th of that observed for the more potent lots.

To compare the potency of these vaccines in mice, duplicate antigen extinction tests were carried out simultaneously. Reference vaccine Lot No. 12 was included in the test. To increase the accuracy of the assay, 2-fold vaccine dilutions were employed instead of the usual 5-fold vaccine dilutions. The results of each of the duplicate assays were in excellent agreement. A 10-fold range in ED50 values was observed among the different lots. Vaccine Lots 7 and 13 having potency values essentially equal to the reference vaccine, Lot 12, were highly immunogenic in human beings. These 2 lots induced over 90% antibody conversions with an average LNI of approximately 3.0. However, with the exception of Lot 11 fluid vaccine, the vaccine lots having a lower potency value than the reference vaccine resulted in a lower antibody response in man. These data indicate that the employment of vaccine lots equivalent in potency to the reference vaccine should result in an antibody conversion rate in man of 90% or greater. Table IV shows the degree of persistency of antibody level in

TABLE IV. SERUM NEUTRALIZING ANTIBODY TITERS IN MAN AT STATED INTERVALS POSTVACCINATION

HUMAN SUBJECT		LOG 10 ANTIBODY TITERS			
No.[a]	(Initials)	14 days	6 mon	12 mon	18 mon
1	(A.B.)	2.8	1.7	1.6	Not done
2	(J.B.)	4.0	2.3	2.1	Not done
3	(G.E.)	3.5	2.2	2.1	Not done
4	(C.T.)	4.0	2.2	1.9	2.7
5	(E.N.G.)	4.0	2.5	1.5	1.8
6	(E.W.J.)	5.2	2.3	2.0	2.6
7	(F.T.W.)	1.8	2.4	0.9	1.8
8	(N.F.M.)	2.2	2.1	1.5	2.1
9	(J.S.G.)	>5.5	3.3	2.0	2.1
10	(R.M.B.)	3.1	2.3	1.5	1.6
11	(J.W.J.)	3.7	2.8	1.6	2.6
12	(H.H.M.)	4.2	2.0	1.1	2.3
13	(H.C.E.)	2.5	2.0	1.4	1.5
14	(H.B.B.)	1.8	2.6	1.5	1.8
15	(C.W.C.)	2.3	2.6	1.6	Not done

a. Each individual received 3 1.0-ml doses of RVF vaccine of the P-MKC type on days 0, 7-10 and 28-35, respectively.

man over an 18-month period following three 1-ml doses of RVF vaccine. Since a log neutralization index of 1.7 or greater is considered to provide ample protection, it may be noted that 9 of the 11 available vaccinees were considered to possess effective protection against infection with RVF virus for at least 18 months postvaccination period.

In order to manufacture commercially and license RVF vaccine, a tentative draft of the "Minimum Requirements" is being prepared for publication in the Federal Register. The late Dr. J. E. Smadel of the National Institutes of Health and Lt Colonel E. W. Grogan, VC, of the U. S. Army Medical Unit, Fort Detrick, have collaborated in the preparation of the "Minimum Requirements."

Several meetings have been held with personnel of the U. S. Army Medical Unit, Fort Detrick, and representatives of the National Drug Company, Swiftwater, Pennsylvania. At these meetings the methods for the preparation and testing of RVF vaccine have been discussed in detail. The Department of Hazardous Operations has provided vaccine for the immunization of the drug company's personnel and is prepared to furnish approved strains of the virus, a lyophilized standard reference vaccine, and an adequate supply of highly potent lyophilized rabbit antiserum free from antibody against lymphocytic choriomeningitis and other adventitious simian viruses.

In summary, the development and production methods for a safe and highly immunogenic RVF vaccine have reached the stage where it is now ready to be produced in large quantities by a commercial source.

List of Publications:

1. Randall, R., Gibbs, C. J., Jr., Aulisio, C. G., Binn, L. N., and Harrison, V. R.: "The Development of a Formalin-Killed Rift Valley Fever Virus Vaccine for Use in Man," J Immunol 89:660-671, 1962.

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3. Randall, R., Harrison, V. R., and Binn, L. N. "The Immunological Response of Human Beings to a Formalin-Inactivated Rift Valley Fever Virus Vaccine," Presented at the 13th Annual Southwestern Conference on Diseases in Nature Transmissible to Man, San Antonio, Texas, 4-5 April 1963.

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REPORT ON PLAGUE ANTIGENS

John D. Marshall, Major, MSC*

Studies by Lawton, et al[1] have reported that Pasteurella pestis contains at least 18 different antigens as demonstrated by Ouchterlony double diffusion tests.

Four of these antigens have been studied in detail by various laboratories. The F antigen (Fraction I) has received the greatest attention. Dr. Meyer and his group[2,3] have categorized it immunologically and chemically, and have shown it to be an effective immunizing agent against experimental plague.

The V and W antigens have been studied by both the Fort Detrick[4] and Porton groups.[5] The V factor has been shown to be an effective immunizing antigen, while the W factor does not appear to elicit any degree of protective immunity in experimental animals.

The T antigen (murine toxin) was purified and categorized by Ajl, et al.[6] This antigen, while altering the course of a plague infection, did not significantly alter the mortality rate.

Burrows and Bacon[7] have shown that P. pestis strains devoid of antigens F, V, and W, when used as vaccines, protected animals against challenge infection with a fully virulent strain. They concluded that there are one or more yet uncategorized protective antigens in P. pestis.

The work initiated in our laboratory is directed toward the isolation, purification and characterization of the remaining antigens. To date, we have employed fractional ammonium sulfate precipitation, column chromatography and discontinous gel or disc electrophoresis for the separation of the antigens. Varying degrees of success have been obtained by these methods.

P. pestis, Strain EV 76, was grown on Casman's agar at 37 C for 4 days, harvested in 10 ml physiological saline. The saline suspension was aspirated into 50 volumes of cole acetone (-70 C) which was then permitted to warm to room temperature. The acetone-killed and dried (AKD) P. pestis was collected by filtration, washed with several volumes of dry acetone, and pooled.

Fifty grams of AKD P. pestis cells were extracted with buffered saline, pH 7.2 for 18 hr at 37 C. The cells were sedimented and the supernatant fluid fractionated with increasing concentrations of ammonium sulfate. Precipitates were collected by centrifugation at 27, 40, 55, 63, 70 and 100% saturation. The precipitates were dialyzed against 0.1 M phosphate pH 7.0 till free of ammonium sulfate. The various fractions were then subjected to column chromatography.

* U. S. Army Medical Unit, Fort Detrick, Maryland.

Four antigen preparations have been obtained in a relatively pure form. Two, when tested against a whole cell hyperimmune serum showed a single major band on Ouchterlony double diffusion. When these 2 preparations were used to immunize rabbits and then tested against the immune sera, Ouchterlony double diffusion showed 2 and 4 precipitin bands. The remaining 2 antigens appeared pure, showing a single band on double diffusion and on disc electrophoresis. Antisera prepared against these second 2 antigens show a single band when tested against the homologous materials and also when tested against the crude 0-27% ammonium sulfate precipitate from which it was purified.

Table I shows the characteristics of the ammonium sulfate precipitates of P. pestis, Strain EV 76.

TABLE I. CHARACTERIZATION OF AMMONIUM SULFATE PRECIPITATES OF SALINE EXTRACT OF AKD P. PESTIS EV 76 AMMONIUM SULFATE PRECIPITATION

SATURATION OF $(NH_4)_2 SO_4$ %	NUMBER OF LINES		
	Ouchterlony Double Diffusion	Smithies' Gel Electrophoresis	Discontinuous Acrylamide Gel Electrophoresis
27	5	3	3
40	7	3	5
55	6	4	6
63	6	4	7
70	7	4	9
100	6	4	9
SUPERNATANT	2	-	3

The total number of lines shown by any of the 3 methods do not represent the number of individual antigens present in the original extract, as several antigens were found in more than one fraction. The cultural conditions used to produce the AKD cells were such as to preclude the formation of either the V or W antigen. Therefore, these antigens are not represented in this preparation.

The discontinuous acrylamide gel electrophoresis technique was found to be more sensitive than Smithies' starch gel electrophoresis for demonstrating differences in electromobility of the various proteins. Further work is in progress to determine the interrelationships between the various bands found by each method.

Table II presents the data obtained from DEAE cellulose chromatography of the 0-27% ammonium sulfate fraction.

TABLE II. DEAE CELLULOSE SEPARATION OF P. PESTIS ANTIGENS

FRACTION NUMBER	NUMBER OF LINES		PROTEIN mg
	Ouchterlony Double Diffusion	Disc Electrophoresis	
1 - 3	0	0	
4 - 6	1	1	10.5
7 - 13	0	0	
14 - 18	1	1	42
19 - 22	0	0	
23 - 35	1	3	8
36 - 48	0	0	

Conditions:

0-27% ammonium sulfate fraction 111 mg protein
DEAE cellulose column 0.01 M Tris chloride buffer pH 8.2
Gradient elution 0.01 M - 1.0 M Tris chloride pH 8.2

The original 0-27% ammonium sulfate fraction shows 5 distinct lines when tested by Ouchterlony double diffusion against whole cell P. pestis antiserum. Ouchterlony tests showed fraction 4-6 to be identical with fraction 23-25. Fraction 4-6 probably represents a wash-through of unbound antigens. Fraction 14-18 showed only one precipitation line; the line crossed both the 4-6 and 23-35 fraction lines.

When these fractions were placed in wells adjacent to crude precipitate, identity reactions were observed between the purified fraction and a single component of the crude ammonium sulfate precipitate.

Figure 1 shows one of the Ouchterlony double diffusion tests. The bottom well contains the 0-27% ammonium sulfate fraction, the upper well the 14-18 DEAE cellulose fraction, the right hand well the whole cell P. pestis antiserum and the left hand well the antiserum prepared against the 14-18 fraction.

FIGURE 1. OUCHTERLONY DOUBLE DIFFUSION TEST OF FRACTION 14–18.

Five precipitin bands are seen between the ammonium sulfate fraction and the whole cell antiserum. A single faint band is present between the 14-18 DEAE cellulose fraction and the whole cell antiserum. A single line can be observed between the 14-18 DEAE fraction and the antiserum prepared against this fraction. The single line between the 14-18 DEAE fraction and the ammonium sulfate fraction forms an identity reaction with the second precipitin line formed by the reaction between ammonium sulfate fraction and the whole cell P. pestis antiserum. The remaining 4 lines either cross or extend past the single band indicating nonidentity.

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